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TITLE: Enhanced Androgen Signaling with Androgen Receptor Overexpression in the Osteoblast Lineage Controls Skeletal Turnover, Matrix Quality and Bone Architecture

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14. ABSTRACT

Androgens have been shown to be important mediators of bone growth and remodeling independent of estrogen. We genetically engineered transgenic mice in which androgen receptor (AR) overexpression is skeletally targeted in two separate models to better understand the role of androgen signaling directly in bone. In the fourth year, we have published the analysis of the second line of AR-transgenic mice, AR2.3-transgenic mice. Enhanced androgen signaling directly in bone results in inhibition of bone formation by differentiated osteoblasts, with a phenotype reflecting low turnover. Comparisons between both models of AR2.3-and AR3.6-transgenic animals suggests that AR transactivation in osteocytes is primarily responsible for mediating the effects of androgen on matrix quality and/or mineralization (inhibitory), while stromal/immature cells mediate effects of androgen on the periosteum and body composition (anabolic). The consequence of androgen action *in vivo* is compartment-specific; anabolic effects are exhibited exclusively at periosteal surfaces, but in mature osteoblasts androgens inhibit osteogenesis with detrimental effects on matrix quality, bone fragility and whole bone strength (Specific Aim 1). Gene expression profiling has identified important signaling pathways by which androgens influence osteoblast-osteoclast communication (Specific aim 2). With immediate androgen replacement during a sustained hypogonadal period, gender differences are observed in the response to androgen replacement: both males and females demonstrate improved bone mineral, but female AR-transgenic mice are insensitive to improvements. These results indicate that direct androgen is generally not anabolic in the skeleton. Gene expression analysis of androgen action in endocortical bone and differentiation in calvarial cultures from AR2.3-tg mice (Specific aim 3) are ongoing. Results indicate regulation of genes involved in osteoblast differentiation that may be detrimental.

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Introduction

Androgen deficiency (as a result of aging, hypogonadism, glucocorticoid therapy, or alcoholism), and other behaviors (chronic smoking, malabsorption and bone marrow malignancies) are associated with the development of osteoporosis in men (1). Osteoporosis is also an important and debilitating side effect of androgen deprivation therapy in conjunction with the treatment of prostate cancer (2, 3). At any one time, osteoporosis affects 20 million Americans. Nearly one-quarter of the patients who suffer a hip fracture die within the first year; 50% of patients are unable to walk without assistance; and 33% are totally dependent (4, 5). Of the 1.3 million bone fractures that can be attributed to osteoporosis every year, 150,000 are hip fractures that occur in men with lifetime risk for the development of fracture at nearly 15% (6). In addition, it is also clear that androgens have an important but very much under-appreciated role in women (7). Other health problems may also be affected by androgen action, including atherosclerotic vascular disease, age-related weakness and disability, memory loss, etc. Since osteoporosis is often coupled with a hypogonadal state, developing an understanding of androgen action in the skeleton may provide insight into development of novel therapeutics for the treatment of osteoporosis and metabolic bone disease.

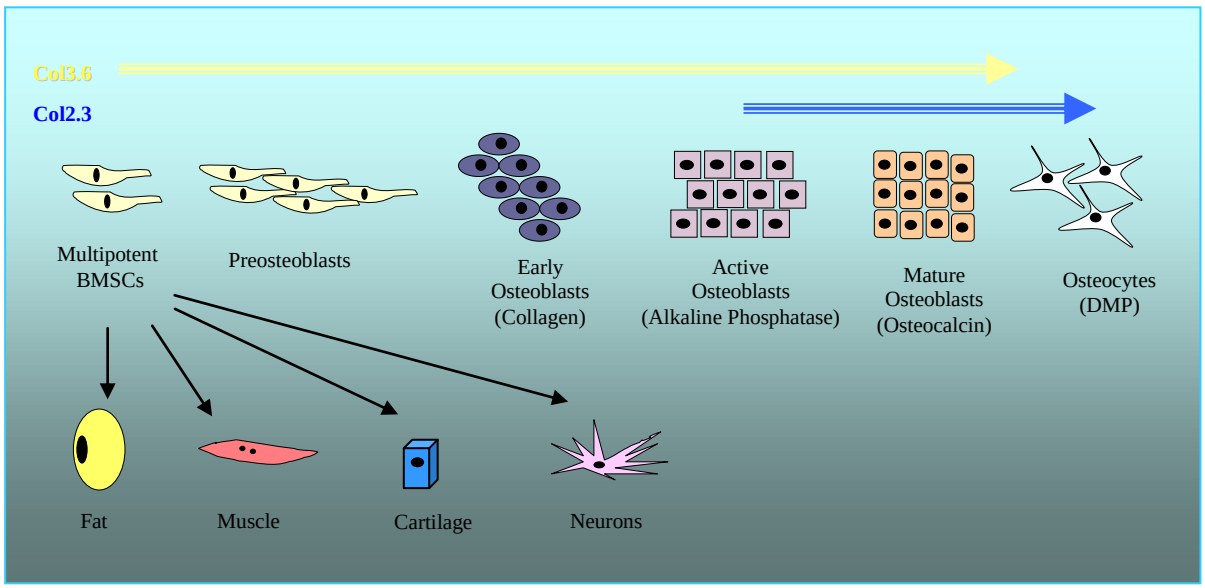
The distinct contribution of androgen to the maintenance of a healthy skeleton remains controversial, since the major androgen metabolite testosterone can serve as the substrate for the production estradiol via aromatase activity. As a consequence, some testosterone action may result from estrogen receptor-dependent activation after conversion to 17-ß estradiol. Overexpression of androgen receptor (AR), combined with the use of non-aromatizable androgens that cannot serve as a substrate for aromatase conversion (e.g. 5α-dihydrotestosterone; DHT), should enhance our understanding of the *specific* role for androgen in bone biology. The goal of this program is to gain a comprehensive understanding of the cascade of molecular and cellular events by which androgen signaling influences skeletal homeostasis.

Our proposed studies have substantial military significance. The stated goals of the Bone Health and Military Readiness program are to advance the understanding of methods to improve bone health of young men and women, to enhance military readiness by reducing the incidence of fracture during physically intensive training, and to reduce the incidence of osteoporosis later in life. As little is known about the direct actions of androgens on osteoblasts, our comprehensive approach using unique animal models of enhanced androgen responsiveness with distinct bone-targeted AR-transgenic families, combined with the novel studies of DHT modulation of osteoblast differentiation and osteoblast-osteoclast signaling, will provide insights into normal bone homeostasis. Understanding the consequences of androgen action in bone is particularly important given increased anabolic steroid abuse. In addition, since bone architecture and bone material properties play important roles in stress fracture, analysis of this model represents a unique opportunity to characterize the consequences of androgen action in both genders on bone microarchitectural quality and the integrity of the skeleton *in vivo*.

Body

In the fourth year of this grant, we have published the characterization of a distinct set of AR-transgenic mice, AR2.3-transgenic families, that were constructed using a smaller collagen promoter fragment to drive AR overexpression in mature osteoblasts (as outlined in Specific Aim 1). Thus, in addition to the AR3.6-transgenic families we have previously characterized with AR overexpression in stromal cells and throughout the osteoblast lineage including mature osteoblasts, the AR2.3-transgenic mice now also provide models for the characterization of enhanced androgen signaling in distinct skeletal compartments through a comparison the

phenotypes observed in both lines (see summary illustration below). Importantly in both models, enhanced androgen action occurs only in those cells with elevated levels of AR (skeletally-targeted) as a consequence of enhanced AR signaling, without changes in circulating steroid levels and without systemic androgen administration. Because of distinct and overlapping expression profiles as shown in the schematic below, comparison of the skeletal phenotypes characterized in these two models of enhanced androgen action are postulated to aid in the identification of cells within the osteoblast lineage that are most important for mediating a specific response in bone modeling/remodeling characteristics. For example, phenotypes identified as similar in both AR2.3- transgenic and AR3.6-transgenic suggests that mature osteoblasts/osteocytes are important mediators of the response, since there is overlap in promoter activity in those cell types. In contrast, phenotypes that are more pronounced in AR3.6- transgenic compared to AR2.3-transgenic suggest that stromal or immature osteoblasts are primary mediators.



Our goal as proposed in Specific Aim 1 is to contrast the skeletal phenotype of AR2.3-transgenic with AR3.6-transgenic animals (with different AR overexpression profiles in the osteoblast lineage), in the adult and in the hypogonadal state in both genders, to identify direct androgen actions *in vivo*. This analysis will allow us to test the hypothesis that distinct profiles of AR overexpression in the osteoblast lineage will result in distinct skeletal phenotypes between AR2.3- transgenic vs. AR3.6-transgenic mice. The progress report is divided into three sections: **(1)**. Studies proposed in Specific aim 1 to compare and contrast the phenotype in AR2.3- transgenic and AR3.6-transgenic lines: a) completion of analysis of the adult AR2.3-transgenic mouse model (manuscript published); b) analysis of DHT replacement studies in both a low and high bone turnover situation after gonadectomy in both males and females in both AR2.3- and AR3.6-transgenic mice for evaluation of bone parameters; c) analysis of body composition changes in low and high turnover gonadectomized mice. **(2)**. Studies proposed in Specific aim 2 to determine the importance of AR in regulating osteoclast formation and activation: a) gene expression profiling employing quantitative RT-PCR array (qPCR) analysis to identify important signal transduction pathways altered in osteocytes that mediate the inhibitory effects of androgen on osteoblast vigor and on osteoclastogenesis and/or osteoclast activity in qPCR array. These studies employed an *in vivo* analysis using whole cortical bone from which the periosteum was stripped, marrow elements flushed and metaphyses removed.

This allowed a focus on osteocyte expression in an *in vivo* system. **(3)**. Studies proposed in Specific aim 3 to characterize the role of androgen in the regulation of osteoblast differentiation: a) characterization of the effects of DHT treatment on proliferation, osteoblast differentiation and mineralization in AR2.3-transgenic calvarial mouse osteoblast (mOB) primary cultures.

Section 1: Studies proposed in Specific Aim 1 to analyze the consequences of enhanced androgen signaling in bone employing AR2.3-transgenic and AR3.6-transgenic models

An important advantage of the both the AR3.6-transgenic and AR2.3-transgenic mouse models are the enhancement of androgen signaling as a consequence of increased AR abundance in likely target (tissues or cells) for androgen *in vivo*, i.e., periosteal cells and the osteoblast lineage compared to mature osteoblasts and osteocytes. These models, characterized by the absence of differences in circulating testosterone or 17ß-estradiol and studied without systemic androgen administration, thus takes advantage of increased sensitivity to androgen in distinct skeletal sites for the analysis of compartment-specific effects of androgen. At the same time, AR overexpression, rather than systemic administration, excludes action at other androgen target tissues *in vivo* including muscle and fat. AR overexpression targeted by the col2.3 promoter was chosen for several reasons: the skeletal expression patterns for this promoter are both well-characterized and bone-selective (see 8, 9); the col2.3 promoter is not active in the periosteum but is strongly expressed in osteocytes and mineralizing nodules (10); the col2.3 promoter is also not active in osteoclasts; and androgens do not inhibit expression from the 2.3 kb promoter fragment (data not shown). Thus, the col2.3 promoter fragment directs expression of the fused transgene in bone, with strong expression still observed at the age of 3 months and even in animals as old as 6 months (10). Reports describing characterization of expression indicated strong expression in cells at osteogenic fronts of parietal bones, but the suture area was negative. In long bones, strong transgene expression was observed in most osteoblasts on endosteal surfaces, and in a large proportion of osteocytes in femurs throughout cortical bone, with no expression seen in periosteal fibroblasts (11). In the trabecular area of metaphyseal bone, strong expression was observed at all developmental stages (10).

1a) *Completion of the analysis of the AR2.3-transgenic mouse model at 2 months of age and comparison to AR3.6-transgenic mice*:

Confusion exists regarding the *in vivo* action of androgens in bone due to i) metabolism to estrogen, ii) because androgen influences many tissues in the body and iii) many months of treatment are required to observe improvement in BMD. The AR2.3-transgenic animal model was created to determine the specific physiologic relevance of androgen action in the *mature osteoblast /osteocyte* population in bone, through tissue-specific overexpression of AR. Complete characterization of the AR2.3-transgenic mice is now published (12). This line is distinct from our previously generated transgenic model with AR overexpression in stomal precursors, periosteal fibroblasts and throughout the osteoblast lineage, the AR3.6-transgenic line (13).

Complete the comparison between AR2.3-transgenic and AR3.6-transgenic models to identify target cells in bone: One significant goal for this project was identification of target cells in the skeleton that mediate responses to androgen. Thus, it is instructive to compare the skeletal phenotypes that develop in the two distinct lines that we have generated, the AR2.3-transgenic mice described in these studies and the previously characterized AR3.6-transgenic model (13). In broad terms, the skeletal phenotype characterized in AR2.3-transgenic mice mirrors that described previously for AR3.6-transgenic males, indicating the specificity and reproducibility of the phenotypic consequences of bone-targeted androgen signaling. In

common between the two models, we have shown increased trabecular bone volume, reduced formation at endosteal surfaces, reduced bone turnover and compromised biomechanical strength in male transgenic mice. With the exception of enhanced periosteal activity in AR3.6-transgenic males as noted below, neither model exhibits anabolic responses in the cortical bone compartment and instead both show inhibition of bone formation at the endosteal surface and compromised biomechanical properties. By comparing and contrasting the two AR-transgenic models, we propose that the commonalities in the bone phenotype between AR2.3-transgenic and AR3.6-transgenic mice arise from AR overexpression in mature osteoblasts and osteocytes, since both promoters are active in these cells. Thus, the increased trabecular bone volume, reduced bone turnover, reduced formation and decreased osteoblast vigor at endosteal surfaces, and compromised biomechanical strength with increased bone fragility observed in both models, are likely to be mediated at least in part by enhanced androgen signaling in mature osteoblasts/osteocytes.

Thus, androgen action in the mature mineralizing osteoblast results in reduced bone biomechanics and dramatically impaired matrix quality, with envelope-specific positive effects on bone formation only at periosteal surfaces. Compared with AR3.6-transgene overexpression throughout the osteoblast lineage (including bone marrow stromal cells, throughout osteoblast differentiation including osteocytes), AR2.3-transgenic males do not show reduction in femur length and demonstrate much less inhibition of whole bone strength properties measured (stiffness, maximum load and work). However, AR2.3-transgenic mice show similar changes indicative of a low turnover phenotype, and similar increases in brittleness (i.e., decreased post-yield deflection), suggesting an analogous change in matrix quality and/or mineralization in both AR3.6-transgenic and AR2.3-transgenic mice. In addition, as we have published, androgen enhances osteoblast apoptosis (14). Thus, one **primary finding in *both* AR-transgenic lines is that androgen signaling in the osteoblast results in inhibition of mineral apposition rate (MAR) indices, strongly suggesting that direct androgen signaling in bone results in reduced osteoblast vigor and, as a consequence, poor matrix quality**.

The most striking contrast between the two AR-transgenic models we have developed is observed at periosteal surfaces in AR3.6-transgenic males, which show increased cortical bone formation in the periosteum and dramatic intramembranous calvarial thickening. This finding was expected, given col3.6 transgene targeting to the periosteum and, conversely, the lack of expression at the same compartment with col2.3 transgene expression. The specificity of the periosteal anabolic effect in AR3.6-transgenic males is consistent with previous reports documenting the importance of androgen signaling in periosteal expansion (15). Thus, we propose that androgen inhibition of medullary bone formation at the endosteal surface in males may subserve an important physiological adaptive function, being key for appropriate spatial distribution and maintenance of the total amount/weight of bone in the cortical envelope. Combined, our data is consistent with the **hypothesis that androgens strongly promote the addition of cortical width through periosteal apposition, but balance that growth with inhibition of bone formation in the marrow cavity so that the skeleton does not become too heavy**. Thus, androgen-mediated **inhibition of bone formation** is a major consequence of AR signaling.

Based on our characterization of AR-transgenic mouse models and other published reports, we have proposed a model for the consequences of androgen signaling where the **effects of AR activation are distinct in different skeletal compartments and can be characterized as envelope-specific** (Fig. 1). Thus, in trabecular bone, androgens reduce bone turnover but increase trabecular volume through an increase in trabecular number. In cortical bone, androgens inhibit osteogenesis at endosteal surfaces but increase bone formation at periosteal

sites (13), to maintain cortical thickness yet displace bone further away from the neutral axis in males. Androgens also positively influence bone at intramembranous sites (13, 16). In addition, androgen administration increases muscle mass, partially mediated by effects on mesenchymal stem cell lineage commitment (17), likely to indirectly influence bone density through biomechanical linkage. Forced exercise in studies employing AR3.6-transgenic mice, with increased lean mass, would help test this hypothesis.

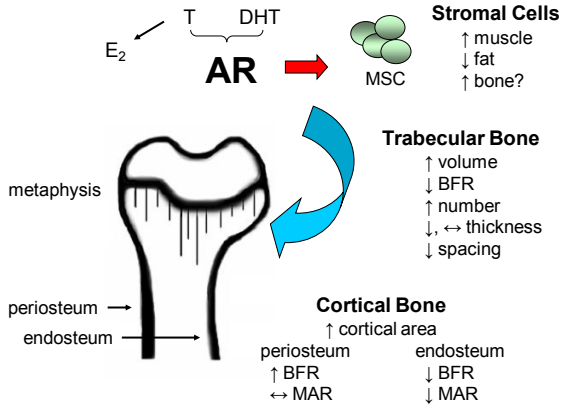


Figure 1. Model for androgen action in the skeleton mediated by AR transactivation. Androgen activation of AR influences a variety of target organs and skeletal sites, including marrow stromal cells, and trabecular, cortical and intramembranous bone compartments. Arrows indicate the changes associated with androgen action. In trabecular bone, androgen action preserves or increases trabecular number, has little effect on trabecular thickness, and thus reduces trabecular spacing. In cortical bone, AR activation results in reduced bone formation at the endosteal surface but stimulation of bone formation at the periosteal surface; correspondingly decreased periosteal but increased endosteal resorption results in no change in cortical area. In the transgenic model, AR activation in mature bone cells *in vivo* results in a low turnover phenotype, with inhibition of bone formation and inhibition of gene expression in both osteoblasts and osteoclasts. In the absence of compensatory changes at the periosteal surface, these changes are detrimental to overall matrix quality, biomechanics and whole bone strength.

To summarize the findings, complex skeletal analysis using morphological characterization by μCT, dynamic and static histomorphometric analysis, DXA, biomechanical testing and gene expression studies all indicate that androgen signaling in mature osteoblasts inhibits osteogenesis at endosteal surfaces and produces a low turnover state; these changes are detrimental to overall matrix quality, biomechanical competence, bone fragility and whole bone strength. It is possible that the observed inhibition of osteogenesis and lack of anabolic response, as a consequence of enhanced androgen signaling in mature bone cells, underscores an important physiological function for androgen in the skeleton: to maintain an appropriate spatial distribution of bone in the cortical envelope. The **strong inhibition of bone formation at the endosteal surface and increase in bone fragility may also underlie the limited therapeutic benefits observed with androgen therapy**. Our studies strongly suggest that because of the detrimental consequences of direct androgen signaling in bone we have documented, anabolic steroid abuse or high dose androgen therapy during growth and in healthy eugonadal adults is potentially damaging.

It should be noted that some of the negative consequences of AR overexpression in mature osteoblasts we have observed *in vivo* are consistent with our in vitro analyses. For example, there are reports, some in clonal osteoblastic cell lines, of effects of gonadal androgen treatment on differentiation, matrix production and mineral accumulation mediated by AR signaling (18-20). These findings are variable however, with other reports of no effect or even inhibition of osteoblast markers (21-23), consistent with our gene expression analysis in AR-transgenic mice. In addition, the effect of androgens on osteoblast proliferation is controversial. We have previously demonstrated that either stimulation or inhibition of osteoblast viability by androgen can be observed, and these effects are dependent on the length of treatment. Transient administration of nonaromatizable DHT can enhance transcription factor activation and osteoblast proliferation, while chronic treatment inhibits both mitogenic signaling and MAP kinase activity (24). Chronic DHT treatment *in vitro* also results in enhanced osteoblast apoptosis (14). In addition, chronic DHT treatment inhibits osteoblast proliferation, enhances osteoblast apoptosis, and suppresses osteoblast differentiation and mineralization (see Figs. 11-15 in Section 3). Thus, *in vitro*

analyses are consistent with the **detrimental changes in matrix quality and dramatic inhibition of osteoblast vigor** we have quantified in both AR-transgenic models *in vivo*.

1b) *Analysis of bone parameters with DHT replacement in adult gonadecomized AR-transgenic mice (both AR2.3 and AR3.6 lines) in the low turnover paradigm:*

A second important goal of our studies was the characterization of androgen signaling in the adult, contrasted with the effects of enhanced signaling we have characterized during skeletal development in the AR2.3- and AR3.6-transgenic mice. With the loss of gonadal steroids (during menopause, andropause or surgical castration), bone turnover is immediately increased dramatically (including increased bone resorption) such that bone strength is reduced. This high turnover state does not persist indefinitely however, and after ~1 year in humans (or ~2 months in mice), turnover rates return to near baseline. We have finished analyses to examine two aspects of the phenotype we have observed in developing AR2.3- and AR3.6-transgenic mice: inhibition of bone turnover (both formation and resorption) at endosteal surfaces (and in trabecular bone), with mild stimulation of bone formation at periosteal surfaces and in calvaria. The hypothesis tested is that DHT transactivation of AR will protect against hypogonadal bone loss following gonadecomy [either ovariectomy (OVX) or orchidectomy (ORX)] in both female and male mice through inhibition of osteoclastogenesis and/or osteoclast activity in wild-type mice. However, we propose that androgen will not display anabolic activity in terms of the osteoblast but will promote bone formation via the periosteal fibroblast. Thus, increases in bone formation rate may be observed exclusively on periosteal surfaces on cortical bone and in calvaria.

Two experimental approaches have been taken to separately test for anti-resorptive and anabolic actions of androgen replacement in the adult, in both AR2.3- and AR3.6-transgenic male and female mice. In the first paradigm presented in last year's progress report, protracted hormone ablation to develop a hypogonadal phenotype was followed by steroid replacement. Both male and female wild-type control (B6D2F2) and AR-transgenic mice were sham operated or gonadectomized at 3 months of age, and the effect of nonaromatizable dihydrotestosterone (DHT) or placebo was determined after an 8-week delay, allowing for gonadectomy-induced changes to develop. In this setting, hormone administration can be considered as a *therapeutic* measure. Following 6 weeks of treatment, the effects of androgen on bone and whole body composition was assessed by DXA. The second approach is characterized as *preventative*. In the second paradigm, hormone ablation as a consequence of gonadecomy at 5 months of age was followed immediately by steroid replacement, again for 6 weeks. Importantly, both groups of animals are analyzed at 6.5 months of age.

In this annual report, we have focused on the second (preventative) paradigm designed to optimize characterization of the anti-catabolic response to androgen signaling, in a model of high turnover. Male and female mice were castrated at ~5 months with immediate steroid pellet replacement. DHT was delivered for ~6 weeks, and mice were then evaluated for changes in bone mineral by dual energy x-ray absorptiometry (DXA) using a mouse PIXImus2 densitometer. Here we present results on the DXA arm of the study. However, our planned histomorphometric analysis was not completed in a timely fashion, and the subcontract for that work has now been awarded to a different laboratory (at Oregon State University). Mouse bones for the high-turnover and low-turnover studies are being transferred to OSU, and histomorphometric and μCT analyses are beginning.

In wild-type (B6D2F2) littermate control animals, six weeks of a hypogonadal state significantly reduced bone mineral density (BMD), bone mineral content (BMC) and bone area in male and female mice (Fig. 2). In wild-type males (n=9-24), ORX resulted in a decrease in BMD by 6.5%, BMC by 12.3% and bone area by ~ 6%. All reductions were significant at $p <$ 0.001. In wild-type males receiving DHT replacement via slow-release pellet during the 6-week period, DHT prevented the loss in BMD by approximately half compared to placebo (2.9% vs. 6.5%, $p < 0.001$). The loss of BMC was also prevented to an extent (8.2 % vs. 12.3%, $p < 0.01$). However, there was no significant improvement in bone area loss. In wild-type females (n = 13-19), the six-week hypogonadal state after OVX resulted in a decrease in BMD by 4.2% with $p <$

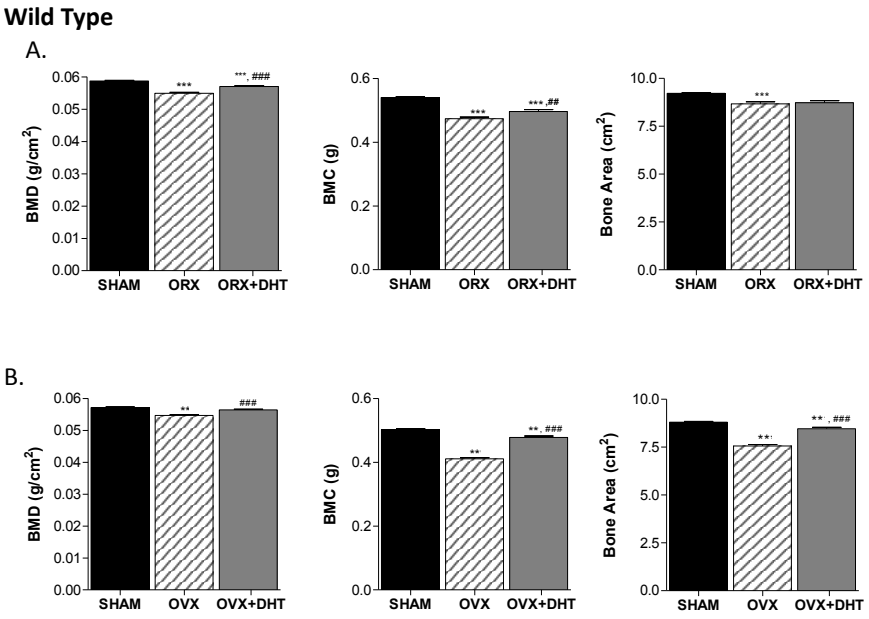


Figure 2. DXA analysis of gonadectomized, wild-type (wt) mice treated with placebo or DHT. ***A***, Male BMD, BMC, and Bone Area (n = 9-24). ***B***, Female BMD, BMC, and Bone Area (n = 13-19). One-way ANOVAs reveal a significant difference of $p < 0.001$. Tukey's multiple comparison test's show significant differences between SHAM and OVX/ORX mice in both genders (** , $p < 0.01$ and *** , $p <$ 0.001) and between DHT administration vs. gonadectomized mice ($^{\#\#}$, $p < 0.01$ and $^{\#\#\#}$, $p <$ 0.001).

0.001, BMC was down 18.25% ($p < 0.001$), and bone area was reduced 14.2 % ($p < 0.001$). Continuous DHT treatment prevented the loss of BMD (only 1.2% with $p < 0.001$ vs. OVX) compared to placebo-treated mice. Similarly, DHT treatment somewhat protected the loss of BMC, down only 5% ($p < 0.001$ vs. OVX), but protection was not completely effective (still $p <$ 0.001 below sham). With DHT, bone area was reduced ~ 3.8%, but was still significantly reduced compared to sham ($p <0.001$). Prevention of bone area loss was still highly significant however ($p < 0.001$). Thus, DHT treatment was sufficient to improve or fully restore these deficits in bone mineral. These results suggest that androgen replacement in hypogonadal populations (either male or female) can be an effective therapy for bone mineral loss particularly in high turnover osteopenia.

Gonadectomy in skeletally-targeted AR-transgenic mice also resulted in a loss of bone

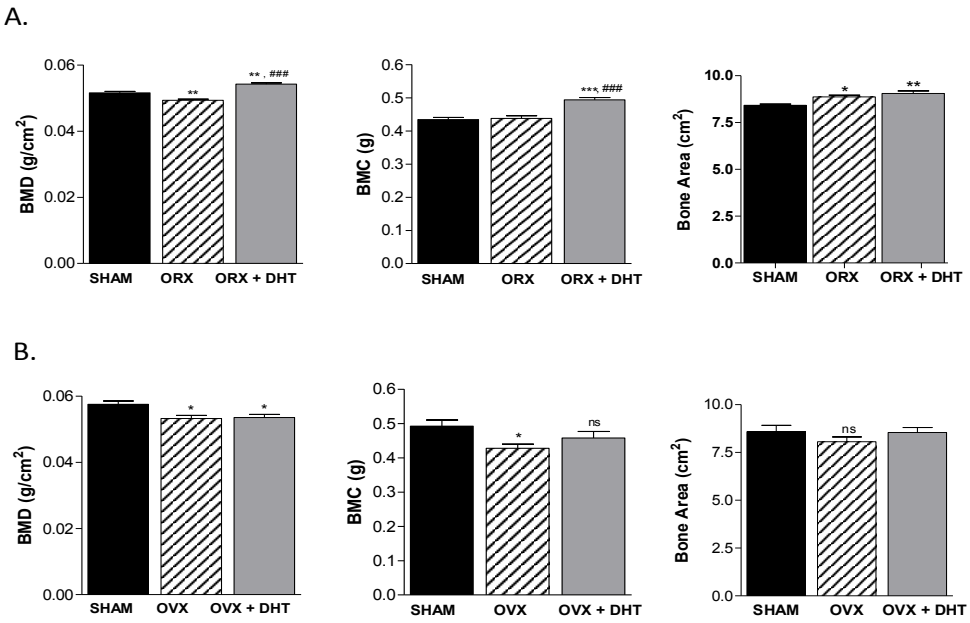


Figure 3. DXA analysis of gonadectomized, AR3.6-tg mice treated with placebo or DHT. ***A***, Male BMD, BMC, and Bone Area (n = 5 - 7). ***B***, Female BMD, BMC, and Bone Area (n = 10). One-way ANOVAs reveal a significant difference of $p < 0.001$ across all measures in males. Female BMD and BMC changes were also significant, $p < 0.01$ and $p < 0.05$, respectively. Tukey's multiple comparison test's show significant differences between SHAM and OVX/ORX mice in some, but not all measures in both genders (** , $p < 0.01$ and * , $p < 0.05$) and between DHT administration vs. ORX in males ($^{###}$, $p < 0.001$).

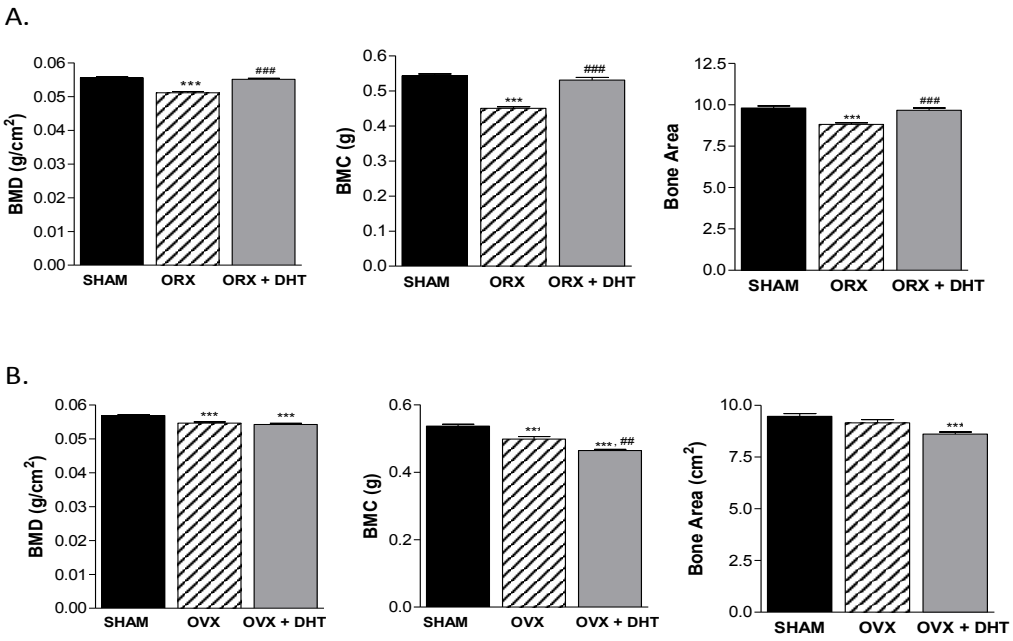


Figure 4. DXA analysis of gonadectomized, AR2.3-tg mice treated with placebo or DHT. ***A***, Male BMD, BMC, and Bone Area (n = 8 - 10). ***B***, Female BMD, BMC, and Bone Area (n = 7 - 11). One-way ANOVAs reveal a significant difference of $p < 0.001$ across all measures in both males and females. Tukey's multiple comparison test's show significant differences between SHAM and OVX/ORX mice in all but female bone area (*** , $p < 0.001$ and ** , $p < 0.01$) and between DHT administration vs. gonadactomized mice ($^{###}$, $p < 0.001$ and $^{##}$, $p < 0.01$).

mineral. However, the response to DHT treatment was blunted or abrogated in females compared to wild-type mice or AR-transgenic males, and the response to DHT was not increased as a consequence of elevated AR abundance (Fig. 3 and Fig. 4). In AR3.6-transgenic males (n = 5-7), BMD was reduced ~ 4.5% ($p < 0.001$) as a result of gonadectomy, and DHT prevented this loss and actually increased BMD by 4.8% above sham controls ($p < 0.001$). BMC did not decrease after ORX, but was low at base-line in this small cohort. DHT

did increased BMC by 11% over sham and ORX mice ($p < 0.001$). Unexpectedly, bone area increased by 5% in ORX mice ($p < 0.05$) and DHT actually increased it further, up to 7% ($p < 0.01$) but again, the number in this cohort is small. In female AR3.6-transgenic mice, BMD decreased 7.5 % after OVX ($p < 0.05$), but there was no effect with continuous DHT treatment. BMC was decreased 13.1% ($p < 0.05$), and there was a trend for prevention of BMC loss, but this was not significant (7% vs. 13.1%). Bone area showed a trend for a small loss and some prevention by DHT (all non-significant differences).

In the AR2.3-transgenic model (Fig. 4), ORX in males (n = 8-10) decreased BMD by ~8% ($p < 0.001$) and DHT prevented this loss with even a small, ~1% increase ($p < 0.001$). BMC decreased 17%, which was also prevented by DHT administration (2% vs. 17%; $p < 0.001$), and bone area decreased 10% compared to only 1.5% with DHT (both $p < 0.001$). In female AR2.3-transgenic mice (n = 7-11), BMD was reduced 4.2% ($p < 0.001$) after OVX, but in contrast to

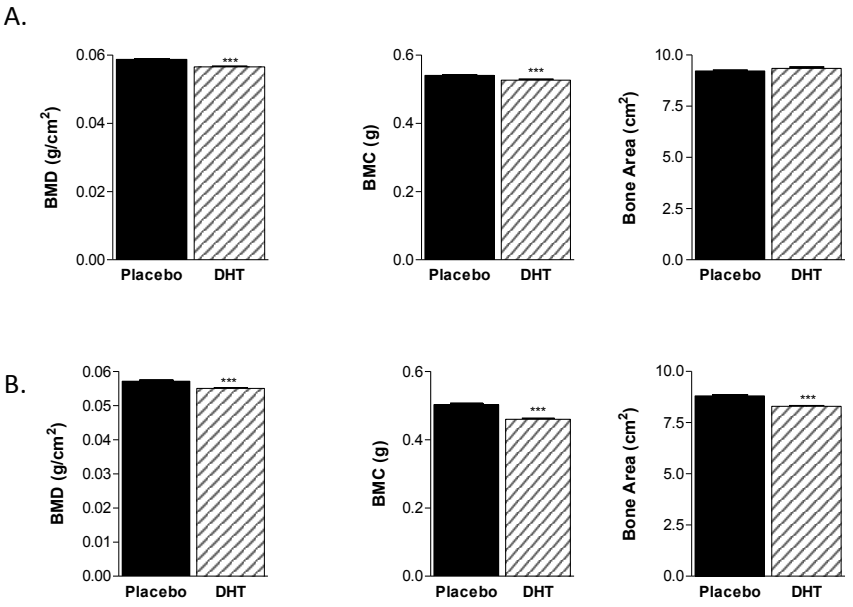


Figure 5. DXA analysis of intact wild-type mice treated with placebo or DHT. ***A***, Male BMD, BMC, and Bone Area (n = 18 - 24). ***B***, Female BMD, BMC, and Bone Area (n = 18 - 19). Statistical analyses by unpaired t-test revealed highly significant losses in bone mineral measures in both males and females (***, p < 0.001), but bone area was only reduced in female mice in this cohort.

males DHT did not prevent bone mineral loss. BMC dropped 7.2% ($p < 0.001$) after OVX, and DHT treatment resulted in a further reduction, down to 13% from sham ($p < 0.01$). Area was not changed by OVX (small non-significant decrease of 3%), but DHT reduced it to 9% of placebo-treated sham controls ($p < 0.001$). This was significantly below the OVX group ($p < 0.05$). Again, as in AR3.6-transgenic females, DHT administration did not reverse the loss in bone mineral as observed in AR3.6-transgenic males or wild type, littermate controls.

Since the response to DHT treatment was not greater compared to wild-type mice, these data suggest that enhanced sensitivity in the osteoblast lineage as a consequence of elevated AR signal transduction does not enhance the response to androgens in the skeleton. In particular, the response of the female AR-transgenic mice was blunted, indicating a gender dimorphism. Effects on bone are being evaluated via histomorphometry and µCT analysis in our subcontract with OSU.

We have also determined the effect of 6 weeks of DHT treatment in intact male and female wild-type mice (Fig. 5). Surprisingly, DHT treatment for six weeks resulted in significant loss of BMD and BMC (p <0.001) in eugonadal males (n = 12-19). BMD was decreased by 3.7% and BMC by 2.7%. Bone area was unchanged. In females (n = 18-19), DHT treatment for six weeks resulted in a significant loss of BMD, BMC and bone area (p <0.001). BMD 3.7%, BMC 8.5 % and bone area was decreased by 5.8% compared with placebo. Feedback regulation of the hypothalamic-pituitary-gonadal axis may partially underlie these results.

Summary of findings for DXA analyses of bone:

- In a model of prevention in a high-turnover setting, nonaromatizable androgen (DHT) effectively ameliorates hypogonadal bone loss in wild-type male and female mice following OVX/ORX.
- In both AR-transgenic lines in the high turnover, prevention model:
    - AR3.6-transgenic mice also lose bone mineral following gonadectomy. Males show an anabolic response to DHT replacement, with increased BMD and BMC. In contrast, female mice do not improve with DHT treatment.
    - Similarly, AR2.3-transgenic male and female mice lose bone mineral following gonadectomy, but DHT treatment ameliorates these changes only in males, similar to wild-type mice.
- DHT administration to intact wild-type mice resulted in detrimental effects on bone, with reduced BMD and BMC in both males and females.

Combined, these data indicate that systemic DHT administration positively influences bone mineral changes in both male and female wild-type controls when present during the period of hypogonadism. Notably, enhanced sensitivity to androgen through AR overexpression in either the mature bone compartment (AR2.3) or in the stromal compartment and proliferating osteoblasts (AR3.6) generally abrogates the positive effects of DHT on bone mineral accretion in males but not female AR-tg mice. Combined, our results suggest that bone, in particular the osteocyte, is not a positive anabolic therapeutic target. Female AR-transgenic mice appear to be insensitive to androgen administration.

1c) *Analysis of body compositional changes gonadectomized mice:*

Androgens have well characterized anabolic effects on muscle mass and strength. In the course of characterization of bone mineral changes in AR3.6-transgenic mice, we noted that male AR3.6-transgenic mice also demonstrate a body composition phenotype, with decreased fat mass but increased lean mass. We have proposed the hypothesis that this change in body composition is a consequence of AR expression in stromal precursors; pluripotential cells with the ability to form a variety of tissues including muscle, fat, bone, and cartilage as outlined in the schema for AR2.3 and AR3.6 expression patterns (Fig. 1). We have previously shown AR3.6-transgene expression in bone marrow stromal cells. With AR transactivation in male mice during development, we propose that bone marrow stromal cells respond by alteration of lineage commitment, away from the adipocyte and instead toward the myoblast to form more muscle in the AR3.6-transgenic mice. DXA analysis has been employed to characterize body compositional changes that occur in the adult after gonadecomy and DHT replacement.

The two experimental paradigms described for bone mineral analysis have been also employed for body composition analysis. In this section, we evaluated potential anabolic (increased lean mass) responses. In our last progress report, we showed data in the delayed

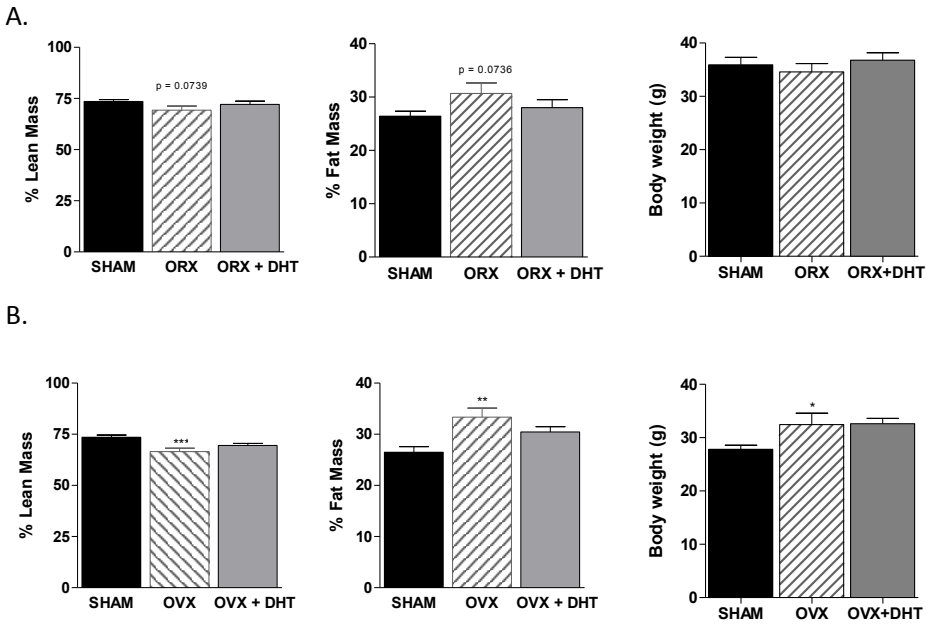


Figure 6. Body composition analysis of gonadectomized wild-type mice treated with placebo or DHT. ***A***, male percent lean mass, percent fat mass (n = 9 - 24), and body weight (n = 9-19). ***B***, female percent lean mass, percent fat mass (n = 13-19), and body weight (n = 13-17). Male mice show trends towards decreased lean mass and increased fat after ORX that are nearly significant, while similar changes in females did reach significance (*, p, 0.05, **, $p < 0.01$, and ***, $p < 0.001$). No significant differences were observed with DHT administration.

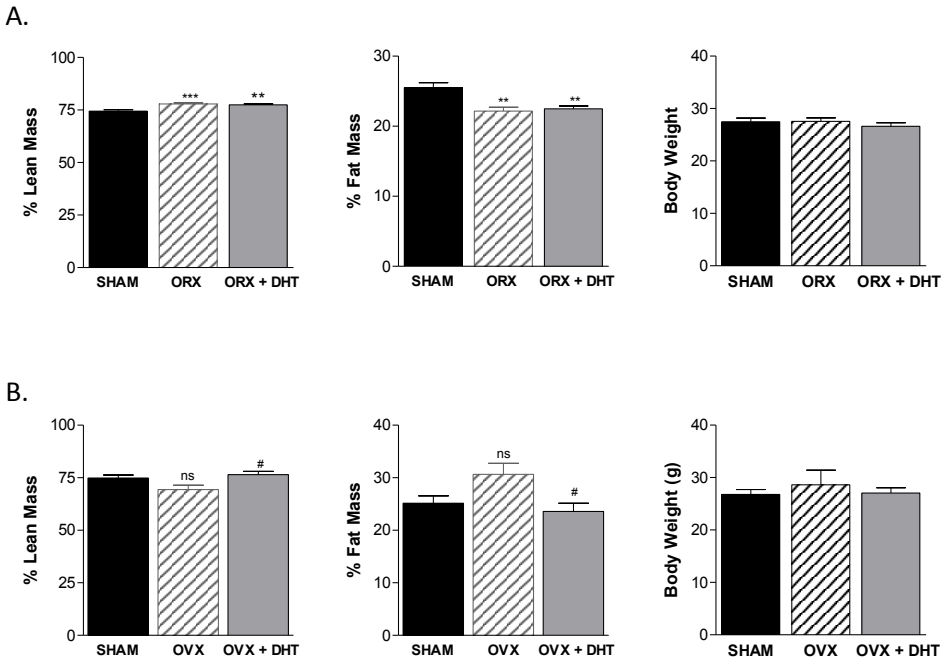


Figure 7. Body composition analysis of gonadectomized AR3.6-tg mice treated with placebo or DHT. ***A***, male percent lean mass, percent fat mass, and body weight (n = 5 - 7). ***B***, female percent lean mass, percent fat mass (n = 10), and body weight (n = 9 -11). One-way ANOVAs reveal significant differences in males with $p < 0.001$ and $p < 0.01$ for lean and fat mass, respectively. Alterations in lean and fat mass in females was significant with $p < 0.05$. No changes in body weight were observed. Tukey's multiple comparison test's show significant differences between SHAM and ORX males (*** , $p < 0.001$ and ** , $p < 0.01$). Trends in OVX females were not significant. Differences following DHT administration in gonadactomized mice was significant only in female lean and fat mass ($^{\#}$, $p < 0.05$).

therapeutic model. Here we show data for male and female mice castrated at 5 months with immediate steroid pellet replacement in the prevention paradigm. DHT was delivered for 6 weeks, and mice were then evaluated for changes in body composition by DXA.

Gonadectomy has been shown to reduce lean mass and increase fat as a percent of body weight in humans and rodent models. Our results show that following ORX, wild-type males showed non-significant trends for reduced lean mass and increased fat mass, and no change in body weight (Fig. 6). In this setting DHT treatment may be beneficial. Although the differences did not quite reach significance, the observed trends indicate improvements in, or prevention of, body compositional alterations with DHT administration. We obtained similar results in wild-type females with a significant loss in lean mass (9.5% with $p < 0.001$) and increased fat (20% with $p < 0.01$) with OVX. Body weight increased 14% ($p < 0.05$). There were trends towards improvement/prevention with DHT, but this did not reach significance. These results suggest that wild-type females may not be as responsive to androgen treatment as an anabolic strategy to increase lean mass.

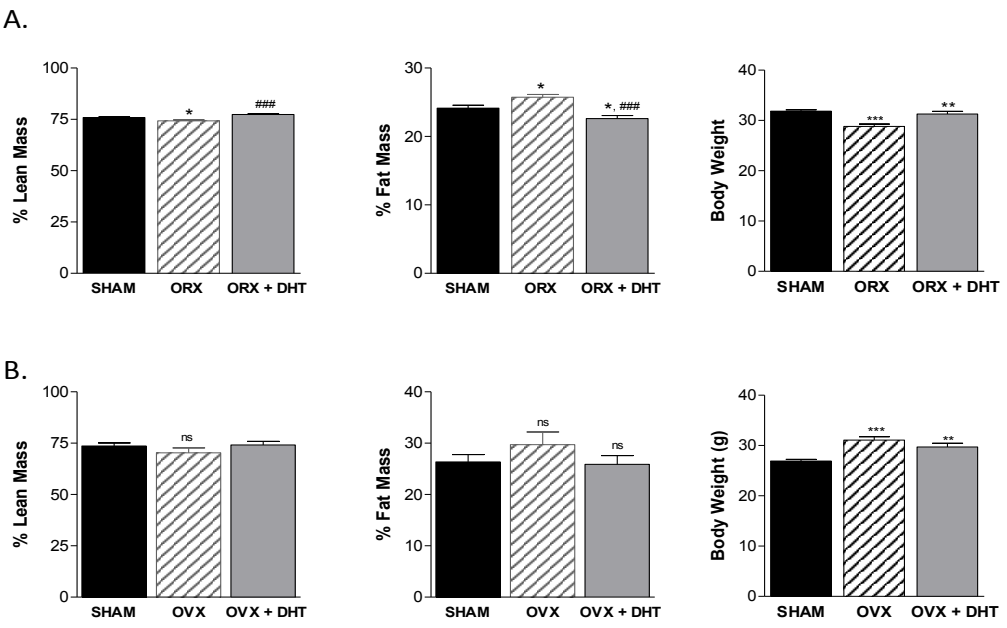


Figure 8. Body composition analysis of gonadectomized AR2.3-tg mice treated with placebo or DHT. ***A***, male percent lean mass, percent fat mass, and body weight (n = 8 - 10). ***B***, female percent lean mass, percent fat mass, and body weight (n = 7 - 11). One-way ANOVAs reveal significant differences in males with $p < 0.001$ across all measures. In females, alterations in lean and fat mass were not significant by one-way ANOVA. Body weight was decreased in males and increased in females (***, $p < 0.001$). For lean and fat mass, Tukey's multiple comparison test's only showed significant differences between SHAM and ORX males (*, $p < 0.05$), while body weight was significant in males and females (*** , $p < 0.001$). DHT administration significantly improved lean and fat mass in males only (**, $p < 0.01$ and ***, $p < 0.001$), although similar trends were observed in females. Body weight changes were also reversed (**, $p < 0.01$).

In general, skeletally-targeted AR overexpression models show similar responses to gonadectomy with the exception of AR3.6-transgenic males (Fig. 7). In this cohort (n = 5-7), lean mass actually increased ~ 4% after ORX ($p < 0.001$). The DHT treatment group was similar to ORX, also above sham-placebo ($p < 0.01$). Fat mass changes were opposite to the effects seen on lean mass, decreasing with ORX and prevented to a small extent with DHT. There were no significant changes in body weight. In AR3.6-transgenic females (n = 10), a non-significant trend for reduced lean mass was observed after gonadectomy (7.5%). DHT prevented this loss and improved lean mass to a level ~ 2% above placebo-treated, sham controls ($p < 0.05$). Fat mass increased 18% with OVX, but this response did not quite reach significance. With DHT treatment during the OVX period however, fat accumulation was entirely prevented, showing a 6% reduction below sham ($p < 0.05$). Body weight changes showed no significant differences.

In AR2.3-transgenic mice (Fig. 8), gonadectomy in males (n = 8-10) resulted in a significant decrease in lean mass (~ 2%; $p < 0.05$). DHT replacement increased lean mass ~2% above sham-operated animals ($p < 0.001$ above ORX). Fat mass increased 6.2% ($p < 0.05$) with ORX, and DHT reduced fat mass ~ 6% from sham and 12% vs. ORX ($p < 0.05$ and $p < 0.001$, respectively). Body weight decreased by 9.5%, yet with DHT the decrease was only 2% ($p < 0.001$ and $p < 0.01$, respectively). In female AR2.3-transgenic mice (n = 7-11), lean mass decreased 4.5 %, but this difference did not reach significance; DHT maintained lean mass at slightly above the sham level, but this was also non-significant. Fat mass increased 11% in OVX females but did not reach significance. DHT reduced fat mass to 1.5% below sham, but again was not statistically significant. Body weight in females was increased 13% after OVX, but the increase was only 9% with DHT ($p < 0.001$ and $p < 0.01$, respectively).

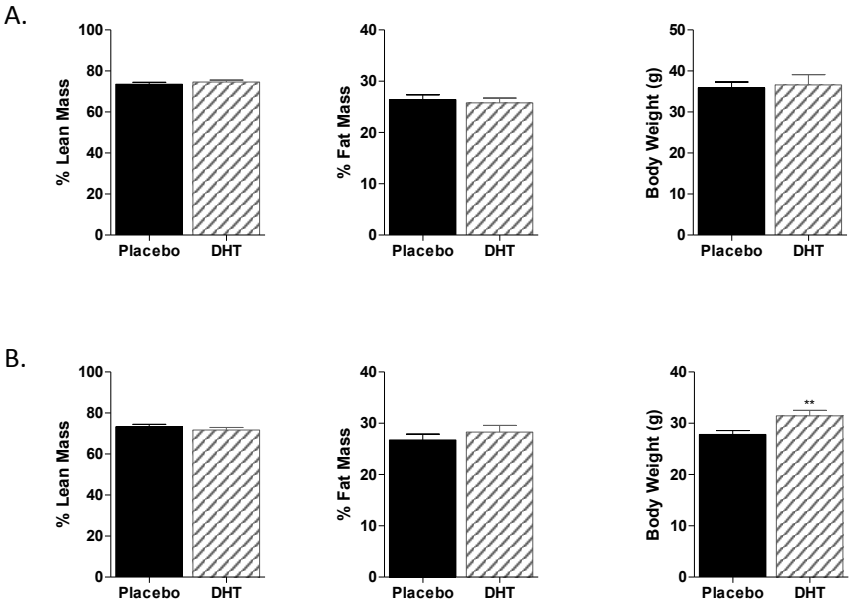


Figure 9. Body composition analysis of intact wild-type mice treated with placebo or DHT. ***A***, male percent lean mass, percent fat mass, and body weight (n = 19 - 24). ***B*,** female percent lean mass, percent fat mass, and body weight (n = 18 - 19). Statistical analyses by unpaired t-test revealed no significant changes in body composition. Females did gain weight however (**, $p < 0.01$).

In contrast to gonadectomized models (Fig. 9), DHT treatment in intact wild-type mice in this cohort showed no significant changes in body composition, although females did gain weight (11.5%, $p < 0.01$).

Summary of findings for body compositional analyses in the high turnover, prevention model:

- In wild-type mice, 6 weeks in a hypogonadal state results in trends for reduced lean mass and increased fat in males, while similar changes were highly significant in females. A trend toward reversal was observed if DHT was present during the hypogonadal period.

- Surprisingly, in a small cohort of AR3.6-transgenic males, mice gained lean mass and lost fat after ORX. DHT treatment did not affect lean or fat mass in these mice. Female AR3.6-tg mice showed nearly significant trends for decreased lean and increased fat mass with OVX, and DHT treatment prevented these changes.

- AR2.3-transgenic male mice showed a significant reduction in lean mass and gain in fat as a result of gonadectomy, and DHT was effective at preventing these changes. AR2.3 females showed similar trends following OVX, changes that were prevented with DHT treatment.

- DHT administration to intact wild-type mice did not affect lean mass or fat mass in either sex, although females did gain body weight.

Combined, these data indicate that systemic DHT administration is effective for the prevention of the body composition changes that result from hypogonadal states in both male and female mice, when present at the onset of the hypogonadal period. In these studies, DHT treatment in the intact animal had no significant effects on body composition. It should be noted that six weeks of treatment may be too short for significant effects on body composition measures using DXA to be observed.

Section 2: Studies proposed in Specific aim 2 to determine the importance of AR in regulating osteoclast formation and activation

Both lines of AR-transgenic mice also demonstrate a phenotype consistent with **reduced osteoclast resorptive activity** in the males. TRAP and RANKL expression is reduced, with an increase in OPG, an important negative regulator of osteoclast differentiation, survival and activation (25). In addition, the increase in trabecular bone volume with a decrease in trabecular separation observed is a hallmark of antiresorptive activity. Potential modulation of osteoclast action by DHT is incompletely characterized, although there are reports of AR expression in the osteoclast (26). The effect of androgen is undoubtedly complex, given data that androgens may inhibit levels of OPG (27, 28), although previously we (13) and others (29) have shown that androgen can stimulate OPG levels. Although androgen may be a less significant determinant of bone resorption *in vivo* than estrogen (30), this remains controversial (31). The bone phenotype that develops in a global AR null male mouse model, a high-turnover osteopenia with reduced trabecular bone volume and a stimulatory effect on osteoclast activity (32-34), also supports the importance of androgen signaling through the AR to influence resorption, and is generally opposite to the phenotype we observe with targeted AR overexpression. Interestingly, the global AR null model also develops late onset obesity (35). Finally, recent publications document that androgen reduces bone resorption of isolated osteoclasts (36), inhibits osteoclast formation stimulated by PTH (37), and may play a direct role regulating aspects of osteoclast activity in conditional AR null mice (38). Our results suggest that at least some component of inhibition of osteoclastic resorptive activity as a consequence of androgen administration is mediated indirectly through effects on mature osteoblasts and osteocytes. These findings reinforce the significance of Specific aim 2 to determine the importance of androgen in mediating osteoclast formation and activation.

2a) *Expression profiling for in vivo characterization of cortical bone samples derived from AR3.6-transgenic mice:*

Although our results suggest that in the AR-transgenic models, osteoclast formation is inhibited, the molecular and cellular mechanisms whereby androgen leads to this complex skeletal phenotype remain unclear and relatively unexplored. Here we sought to identify alterations in molecular signatures as well as functional consequences of increased androgen responsiveness in osteoblastic cells. We first characterized differences between male AR-overexpressing mice and their wild-type counterparts in the expression patterns of osteoblast-

associated genes in cortical bone samples from which both periosteum and marrow elements had been removed. Based on these results we then carried out functional characterizations of osteoblastic cells isolated from wild-type and AR-overexpressing animals (see Section 3).

Gene profiling analyses to characterize pathways mediating androgen inhibition of cortical bone formation.

To gain a better understanding of the transcriptional basis for the inhibition of osteoblast function as a consequence of androgen signaling and to identify important pathways that are physiologically relevant, we used gene profiling analysis. Expression analysis characterized differences in long bones harvested from male mice, using the AR3.6-transgenic line as a source to identify androgen-regulated transcripts in bone because the phenotype is more robust (12, 13). Endocortical bone samples were isolated from tibia after stripping the periosteum, flushing the marrow cavity and removal of the metaphyses, and total RNA was harvested. We have also harvested periosteal samples from these bones for similar expression profiling work. The analysis for periosteal (potentially anabolic) signaling is currently proceeding. Gene expression was surveyed using qPCR arrays for identification of regulated transcripts, with a targeted qPCR array containing 384 genes. Analysis of expression differences using this quantitative platform is believed to be more reliable than standard hybridization approaches. Because little is known about androgen regulation of gene expression in bone, we constructed the array to contain pathways with established importance in bone development and bone remodeling, including with amplicons for genes with previously well-characterized roles in Osteoporosis, TGF-β Signaling and NFκB Signaling, and Targets of Wnt/β-catenin Signaling.

Of the 384 array genes examined, expression of a total of 78 genes (20%) were significantly different between wild-type and AR-transgenic bones (hormone-dependent in the males). Up-regulated sequences are listed in Table 1 and down-regulated sequences are listed in Table 2. We used two independent computational approaches to analyze expression differences: canonical pathways were identified using Ingenuity Pathway Analysis (IPA) software and biological processes were characterized using Pathway Architect software for analysis. As shown in Fig. 10A, canonical pathways were assigned using IPA analysis and the four most significantly regulated were TGFß signaling (p = 2.2E-12), acute phase response signaling (p = 9.5E-08), Wnt/ß-catenin signaling (p = 8.61E-07), and IL-6 signaling (p = 1.31E-06). The pathway that was most significantly modulated was TGFß signaling, which would include genes for TGFß2, Smurf1, Inhibin beta C, Smad6, Smad3, zing finger FYVE domain containing 9, Runx2, fos, jun, six3 and serpine1. Interestingly, the molecular and cellular function most significantly regulated was cell death (p = 1.90E-18).

To characterize biological processes that were impacted by androgen signaling in mature osteoblasts/osteocytes, we employed a second independent analysis suite with Pathway Architect software (Fig. 10B). Examination of the results revealed several notable findings, including negative regulation of osteoblast differentiation, regulation of mineralization, **inhibition of osteoclastogenesis/activity** and regulation of chondrogenesis, all identified as biological themes targeted by androgen. Genes are clustered in boxes relative to a specific signaling cascade, with the first group of genes involved in NFκB signaling, the center cluster involved in TGFß/BMP signaling and the last set are genes that are targets of Wnt/β-catenin signaling. Thus, **regulation of osteoclastogenesis and/or activity by androgen signaling in osteoblasts likely involves changes in IL-6 signaling and NFκB signaling, including the RANK/OPG axis**. In addition, these results prompted us to examine more closely androgen-associated changes in osteoblast function.

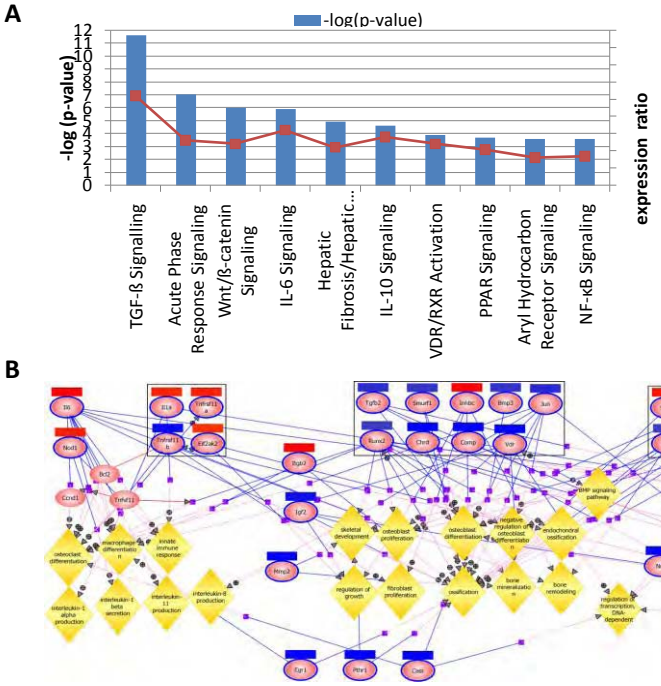


Figure 10. Bioinformatic analyses of androgen-regulated gene expression in cortical bone from AR-transgenic males. ***A***. Summary of signal transduction cascades significantly regulated by androgen signaling in cortical bone. ***B***. Characterization of biological processes. Upregulated transcripts are blue; downregulated are red. Biological processes are indicated by yellow diamonds. Genes involved in NFκB signaling are grouped in a box on the left, genes involved in TGFß/BMP/Activin signaling are grouped in the middle, and genes characterized as targets of Wnt/ß-catenin signaling are on the right. Some genes are influenced by multiple pathways.

Table 1. Upregulation of expression in array qPCR analyses in male transgenic mice from cortical bone samples

Rank	Gene Symbol	Gene Name	Change (fold/control)	p-value
1	Casr	calcium-sensing receptor	19.2768	1.12E-4
5	Sox9	SRY (sex determining region Y)-box 9	3.0469	0.0015
6	Pthr1	parathyroid hormone 1 receptor	2.7079	0.0015
7	Fzd8	frizzled homolog 8	2.8778	0.0016
10	Dcn	decorin	3.1945	0.0019
11	Nrcam	neuronal cell adhesion molecule	3.3979	0.0023
14	Ar	androgen receptor	5.5706	0.0046
18	Cyr61	cysteine rich protein 61(CCN family)	2.2601	0.0054
19	Tcf7l2	transcription factor 7-like 2, T-cell specific, HMG-box	2.1839	0.0059
20	Vdr	vitamin D receptor	2.0357	0.0062
21	Egr1	early growth response 1	2.2339	0.0064
23	Tcf4	transcription factor 4	1.9083	0.0073
24	Inha	inhibin, alpha	3.0395	0.0084
29	Mmp2	matrix metallopeptidase 2	2.2448	0.0103
30	Tnfrsf11b	tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	1.9400	0.0115

32	Col1a2	collagen, type I, alpha 2	2.5745	0.0139
33	Nov	nephroblastoma overexpressed gene	14.0786	0.0139
34	Col1a2	collagen, type I, alpha 2	2.6410	0.0142
36	Chrd	chordin	2.1462	0.0154
37	Tgfb2	transforming growth factor, beta 2	1.7154	0.0156
38	Twist1	twist gene homolog 1	1.7771	0.0156
39	Col1a1	collagen, type I, alpha 1	2.8220	0.0163
44	Igf2	insulin-like growth factor 2	1.8797	0.0184
45	Ppp1r13l	protein phosphatase 1, regulatory (inhibitor) subunit 13 like	1.8986	0.0189
46	Serpine1	serine (or cysteine) peptidase inhibitor, clade E, member 1(Plasminogen activator inhibitor-1 precursor)	1.7626	0.0195
49	Lrp5	low density lipoprotein receptor-related protein 5	1.6801	0.0208
51	Tgfb2	transforming growth factor, beta 2	1.5908	0.0214
52	Fos	FBJ osteosarcoma oncogene	1.6926	0.0220
53	Smurf1	SMAD specific E3 ubiquitin protein ligase 1	1.5902	0.0224
54	Bmp8a	bone morphogenetic protein 8a	1.9434	0.0235
55	Jun	Jun oncogene	1.6677	0.0235
58	Col1a1	collagen, type I, alpha 1	2.5633	0.0251
59	Axin2	axin2	1.8559	0.0255
61	Thbs2	thrombospondin 2	2.1134	0.0260
62	Nppb	natriuretic peptide precursor B	2.3292	0.0271
63	Jun	Jun oncogene	1.6804	0.0275
64	Zfyve9	zinc finger, FYVE domain containing 9	1.5761	0.0295
67	Runx2	runt-related transcription factor 2 (CBFA1)	1.5673	0.0316
68	Fos	FBJ osteosarcoma oncogene	1.6025	0.0326
69	Runx2	runt-related transcription factor 2 (CBFA1)	1.5480	0.0342
70	Comp	cartilage oligomeric matrix protein	6.4464	0.0363
72	Runx2	runt-related transcription factor 2 (CBFA1)	1.4981	0.0377
77	Bmp3	bone morphogenetic protein 3 (osteogenin)	1.5941	0.0431
79	Smad6	MAD homolog 6	1.4881	0.0435
82	Sost	sclerostin	1.5121	0.0454
83	Cyp17a1	cytochrome P450, family 17, subfamily A, polypeptide 1 (steroid 17-alpha-hydroxylase/17,20 lyase)	1.4609	0.0459
85	Fhl2	four and a half LIM domains 2	1.6329	0.0474

Results show genes with significant expression changes when comparing samples after global normalization. Genes are ranked in order of significant changes and listed where the expression change (fold/control) between transgenic and wild-type control was significant at $p < 0.05$, calculated with respect to normalizers based on the GPR algorithm. Analysis was from two samples in replicate representing both AR3.6-transgenic families.

Table 2. Downregulation of expression in array qPCR analyses in male transgenic mice

Rank	Gene Name	Change (fold/control)	*p*-value
2	inhibin, beta C	-10.2696	1.456E-4
3	melanin-concentrating hormone receptor 1	-21.2407	3.296E-4
9	T-cell lymphoma invasion and metastasis 1 (a guanine nucleotide exchange factor that activates Rac)	-2.6613	0.0019
12	Neurogenin 1	-13.8407	0.0040
13	Baculoviral IAP repeat-containing 5 (member of the inhibitors of apoptosis proteins)	-2.1937	0.0046
15	Growth differentiation factor 3 (TGFß superfamily)	-2.2574	0.0047
16	Integrin, beta 2	-2.3143	0.0047
17	Interleukin 6	-3.3141	0.0054
22	Ataxia telangiectasia mutated homolog	-1.9016	0.0070
25	Von Willebrand factor homolog (endothelial cell marker)	-2.0135	0.0088
26	ATP-binding cassette, sub-family B (MDR/TAP), member 1B (multidrug resistance phosphoglycoprotein)	-2.1308	0.0093
27	Sine oculis-related homeobox 3 homolog (inhibition of BMP4)	-1.9793	0.0099
28	TNFRSF1A-associated via death domain	-1.8948	0.0101
31	Caspase 1	-1.8642	0.0129
40	Tumor necrosis factor receptor superfamily, member 11a (RANK)	-1.7563	0.0169
41	pro-opiomelanocortin-alpha	-1.7018	0.0172
42	interleukin 1 alpha	-1.7158	0.0175
43	NF-kappaB repressing factor	-2.0778	0.0182
47	arachidonate 15-lipoxygenase	-1.6693	0.0199
50	nucleotide-binding oligomerization domain containing 1	-1.7473	0.0214
56	secreted frizzled-related protein 4	-1.8160	0.0240
57	eukaryotic translation initiation factor 2-alpha kinase 2	-1.5912	0.0248
60	histidine decarboxylase	-1.6997	0.0255
65	beta-transducin repeat containing protein	-1.7723	0.0299
66	E2F transcription factor 4	-1.5967	0.0309
71	inhibitor of kappaB kinase epsilon	-2.0372	0.0377
73	caspase 8	-1.5088	0.0393
74	receptor (TNFRSF)-interacting serine-threonine kinase 2	-1.4677	0.0397
75	caspase recruitment domain family, member 11	-1.4977	0.0413
76	Tnf receptor-associated factor 3	-2.6919	0.0431
78	TRAF family member-associated NF-kappa B activator	-1.5087	0.0433
80	v-rel reticuloendotheliosis viral oncogene homolog (avian)	-2.1804	0.0444
81	WNT1 inducible signaling pathway protein 2	-1.8771	0.0445
84	beta-transducin repeat containing protein (an F-box protein)	-1.5384	0.0466

Analysis was as described in Table 1.

Section 3: studies proposed in Specific aim 3 to characterize the role of androgen in regulating proliferation and differentiation in the osteoblast lineage

3a) *Characterization of proliferation and differentiation in AR2.3-transgenic calvarial osteoblast (mOB) cultures:*

Bioinformatic analyses indicate that androgen signaling in bone is predicted to inhibit osteoblast proliferation and differentiation. Since the array analysis was derived from whole bone samples, some changes identified may have been a consequence of adaption to altered bone morphology and not direct androgen regulation. In order to identify the cell autonomous effects of direct androgen signaling in osteoblasts, we characterized the effects of DHT

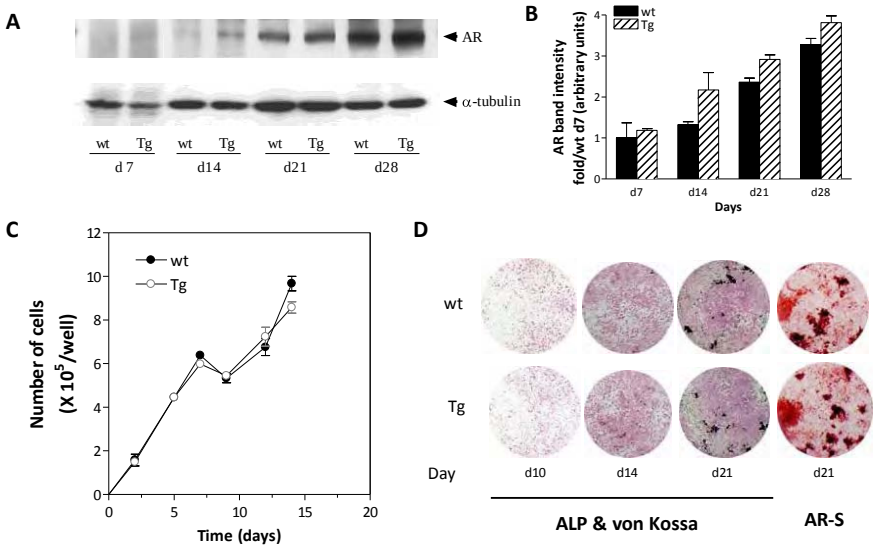


Figure 11. Increased AR levels with osteoblast differentiation and with AR2.3-transgene expression but no effect of AR2.3-transgene expression without hormone addition on osteoblast growth or differentiation. AR abundance was determined in primary osteoblast cultures from AR2.3 mice. Cell extracts were separated on 10% SDS-polyacrylamide gels and AR protein levels were analyzed by Western blotting using polyclonal anti-AR antibody**.** ***A***. Time course analysis of AR protein levels. The upper panel shows AR levels observed in cell lysates of wild-type (wt) and AR2.3-transgenic calvarial osteoblasts (Tg) at indicated time points. The typical AR is indicated by the *arrow* at 110 kDa. Differentiation of primary calvarial cells shows increasing intensity of AR levels over time. The lower panel shows lysates reprobed with α-tubulin antibody in order to assess consistency of protein loading. ***B***. Quantitative analysis of AR abundance was performed by volume densitometry. Data are presented as protein/α-tubulin ratio normalized to expression values at wild-type day 7, followed over the time-course of *in vitro* differentiation. Data are expressed as mean ± SEM **C.** Growth characteristics of untreated wild-type and AR2.3-transgenic osteoblasts. Cells were counted using a Coulter Counter at indicated time points. Data are expressed as mean ± SEM of triplicates. Each experiment was repeated three times. **D**. Differentiation potential of untreated wild-type and AR2.3-transgenic osteoblasts. Cultures were stained for ALP expression on days 10, 14, and 21 by histochemical analysis, and mineralization potential was assessed by von Kossa over ALP staining on days 14 and 21 and by alizarin red S (AR-S) staining on day 21. There was no difference in ALP staining or mineral apposition between wild-type and AR2.3-transgenic osteoblastic cells.

treatment in primary cultures during in vitro differentiation. We focused on AR2.3-transgenic cultures with overexpression in mature osteoblasts/osteocytes because endogenous AR levels are highest in osteocytes. Primary cultures permit a more detailed analysis of the consequences of AR signaling through a comparison between wild-type and AR-transgenic phenotypes. As shown in Fig. 11(A and B), we first evaluated the level of AR abundance in primary calvarial osteoblast cultures derived from wild-type and AR2.3-transgenic mice by Western analysis. AR levels increase during differentiation with the highest levels in the most mature osteoblast/osteocyte cultures, as we have shown previously (39). The influence of the

AR transgene is seen in more mature cultures, with the highest level of AR overexpression noted after day 21, consistent with col2.3 promoter activity (11).

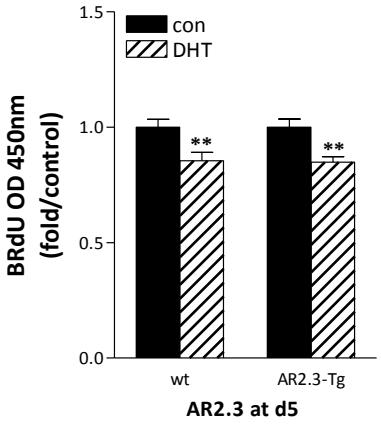


Figure 12. Androgen-mediated inhibition osteoblast proliferation. Primary osteoblasts were cultured from wild-type and AR2.3-transgenic mice in the absence or presence of DHT (10^{-8}M). Changes in proliferation were measured using BrdU incorporation. Cultures were grown in 96-well dish and pulsed for 20 hours with BrdU. Measurements were performed on six wells for each genotype or treatment and expressed as fold vs. vehicle control. DHT significantly reduced osteoblast proliferation in both wild-type and transgenic cultures. Data are expressed as mean ± SEM. **, $p <$ 0.01 compared to exposure to vehicle.

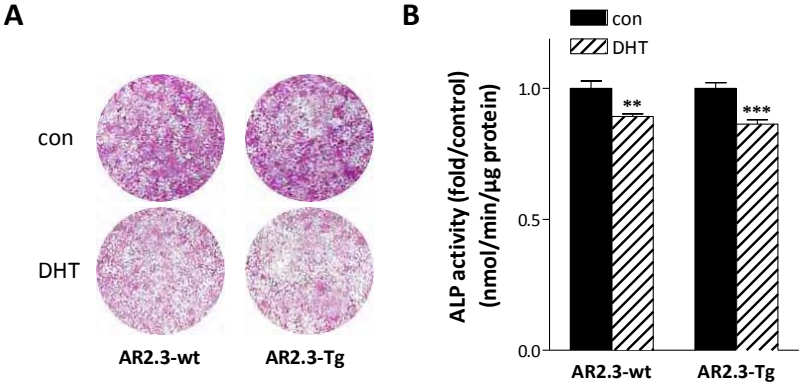


Figure 13. Androgen-mediated inhibition of alkaline phosphatase (ALP) during differentiation in *ex vivo* cultures from wild-type and AR2.3-transgenic mice. Primary osteoblasts were cultured in the absence or presence of DHT (10^{-8}M). Changes in alkaline phosphatase were measured after DHT treatment for 14 days in both wild-type and AR2.3-transgenic calvarial osteoblasts. ***A***. ALP staining. ***B***. ALP activity. Alkaline phosphatase activity was expressed as fold vs. vehicle control. Data are expressed as mean ± SEM of six replicates. Each experiment was repeated four times. A significant decrease in ALP activity was demonstrated with DHT treatment. **, $p < 0.01$; ***, $p < 0.001$ compared to exposure to vehicle.

We assessed growth characteristics of wild-type and AR2.3-transgenic cultures without hormone treatment. As shown in Fig. 11C, osteoblastic cells from both transgenic and wild-type mice display similar growth kinetics. We also determined mineralization capacity (Fig. 11D), and observed no significant difference between AR2.3-transgenic versus wild-type cultures without hormone addition. This was expected because without androgen present, AR activation would not occur and thus overexpression is without consequence.

The effect of androgen on osteoblast proliferation, differentiation and mineralization were then characterized, based on biological processes identified in the array analysis. Primary cultures of wild-type and AR2.3-transgenic mice were treated continuously with vehicle or 10^{-8} M DHT (dihydrotestosterone, a nonaromatizable androgen) in charcoal-stripped serum for 5 days. The effect on osteoblast proliferation was assessed by BrdU incorporation into DNA. As shown in Fig. 12, androgen treatment significantly inhibited osteoblast proliferation. There was little difference between wild-type and transgenic culture in the response to androgen since AR levels are similar at this early stage of differentiation.

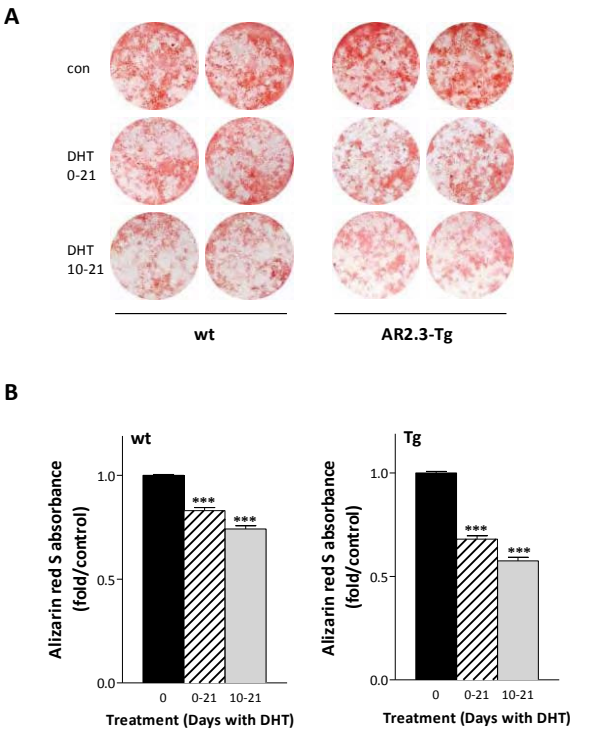


Figure 14. Inhibition of osteoblast mineral deposition after DHT treatment in primary cultures from wild-type and AR2.3-transgenic mice. Osteoblast-enriched primary cultures were derived from fetal calvaria by collagenase digestion. Cells were grown with or without DHT (10^{-8}M) added on the indicated treatment days and isolated on day 21, and mineralization was assessed by alizarin red-S staining (***A***) and alizarin red-S assay (***B***). Data are expressed as mean ± SEM (n = 9). Each experiment was repeated three times. A representative image for AR-S staining was shown. A significant decrease in AR-S activity was demonstrated with DHT treatment. ***, $p < 0.001$ compared to exposure to vehicle.

To characterize the effects of androgen treatment on osteoblast differentiation, we characterized alkaline phosphatase activity, an osteoblast marker (Fig. 13). Cultures were treated continuously with vehicle or 10^{-8}M DHT in charcoal-stripped serum. Cultures treated with DHT showed significant inhibition in alkaline phosphatase activity, with the most robust inhibition observed in AR2.3-transgenic cultures.

We next evaluated the consequence of androgen treatment on mineralization in primary osteoblasts. Cultures were treated with vehicle or 10^{-8}M DHT in charcoal-stripped serum. DHT was added continuously, or at the end of differentiation. Cultures treated with DHT demonstrated inhibition of mineralization assessed by alizarin red-S staining (Fig. 14A). Quantitation showed significant inhibition at all time points, with the largest inhibition observed when androgen was present the last 10 days of culture (Fig. 14B). Mineral nodule formation was determined in primary cultures from AR2.3-transgenic mice after both ALP staining and von Kossa silver staining. Nodule formation was significantly inhibited by DHT treatment (Fig. 15).

Changes in matrix protein gene expression levels were assessed by qPCR analysis (Fig. 16). Primary cultures were treated with DHT continuously, and total RNA isolated at day 14 and day 21. Alkaline phosphatase were assessed at day 14 during matrix maturation, and osteocalcin and SOST were measured at day 21 as mineralization is initiated. DHT treatment inhibited alkaline phosphatase and osteocalcin steady-state gene expression but increases SOST levels.

3b) *Characterization of the differentiation potential of AR3.6-transgenic bone marrow stromal cells in ex vivo culture.*

Finally, we have begun to develop expertise in isolation and differentiation of marrow stromal cells. Bone marrow stromal cells (BMSC) or mesenchymal stem cells are a pluripotent population of cells found at a frequency of 0.5 %-1% in bone marrow. Depending on the signaling milieu of growth factors and hormones received, BMSC will differentiate into multiple lineages including the myoblast, osteoblast, and adipocyte. AR3.6-transgenic lines will be the most informative source of stromal cells because, as we have shown previously, marrow cells show expression of the AR-transgene in this line. These studies will help to characterize the effects of androgen signaling on lineage commitment, and how androgen influence osteogensis.

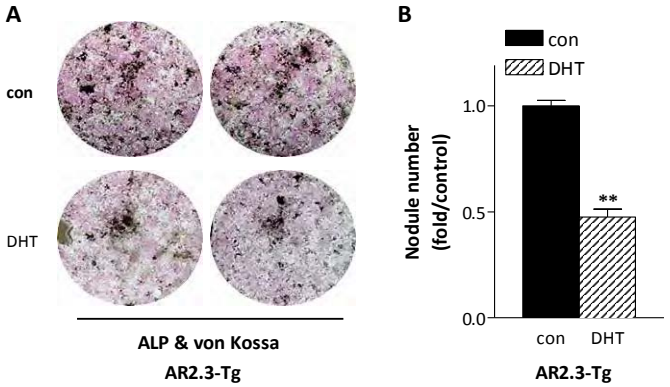


Figure 15. Von Kossa silver staining of mineralized bone nodules over ALP histochemical analysis. ***A.*** Calvarial osteoblastic cells from AR2.3-transgenic mice were cultured with or without DHT (10^{-8}M) for 21 days. The cultures were stained by ALP and von Kossa silver. A significant decrease in both ALP staining and bone nodules was demonstrated with DHT treatment. ***B.*** Quantification of bone nodules formed. Results are expressed in histogram as mean ± SEM of triplicates. A significant decrease in the number of bone nodules was observed with DHT addition. **, p<0.01.

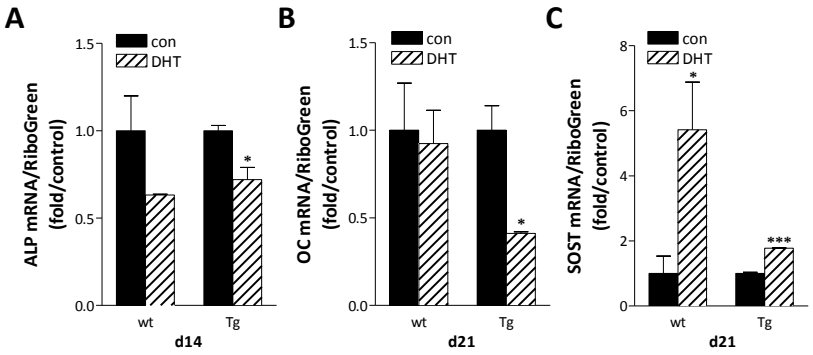


Figure 16. Gene expression characterized in RNA isolated from calvaria in wild-type and AR2.3-transgenic mice with or without DHT treatment. Analysis of steady-state mRNA expression of osteoblast genes was determined by real-time RT-PCR analysis using calvaria RNA isolated from 3-6-day old wild-type or AR2.3-transgenic mice. Osteoblast genes involved in matrix production and mineralization included alkaline phosphatase (ALP), osteocalcin (OC), and sclerostin (SOST). **A.** ALP gene expression in wild type and AR2.3-transgenic calvarial osteoblasts at day 14. **B.** Analysis of OC expression at day 21. **C.** Analysis of SOST expression at day 21.

As shown in Fig. 17, our initial studies have begun analysis to determine whether AR overexpression influences the ability of stem cells to differentiate along a mesenchymal lineage. BMSCs were isolated from wild-type and AR3.6-transgenic male mice. Femurs were harvested, stripped free of muscle, the metaphases were removed and the bone marrow was collected by centrifugation or was flushed using a syringe and 27 g needle. A single cell suspension was plated in 6-well plates at 1.5 x 10^6 cells per well for CFU-F (and 2.5 x 10^6 cells/well for CFU-Ad and CFU-OB) in α-MEM containing 15% fetal calf serum. After six days in culture, half of the media was replaced and the cells were grown for an additional 4-6 days. On day 10-12, cultures were washed, fixed with 4% paraformaldehyde and stained with Giemsa. Digital images were captured and the number of CFU-F colonies were enumerated. This preliminary experiment was performed in duplicate and a total of 12 wells were counted for both wild-type and AR3.6-tg on day 12 of culture. There were no significant differences in the capacity of marrow stem cells to form colonies between wild-type and AR3.6-transgneic mice.

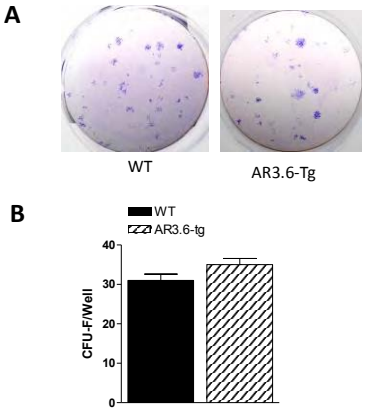


Figure 17. Isolation of marrow stem cells and mesenchymal colony formation capacity. Marrow was harvested from wild-type and AR3.6-transgenic male mice. Cells were plated and grown for colony forming unit (CFU) determination. ***A***. Staining of colonies. ***B***. Quantification of CFU per well from each genotype. There were no significant differences in CFU between wild-type and AR3.6-transgenic mice in these initial studies.

Summary of important results: The specific role of the AR in maintenance of skeletal homoeostasis remains controversial. To determine the specific physiologic relevance of androgen action in the mature osteoblast/osteocyte population in bone, mice with targeted AR overexpression in mature osteoblasts were developed to compare and contrast with transgenic lines with AR overexpression in stromal cells and throughout the osteoblast lineage.

Characterization of the consequences of bone-targeted overexpression revealed a skeletal phenotype in male transgenic mice versus littermate controls, with little difference between the females. Collectively, the phenotype observed in male transgenic mice is likely dependent on the higher levels of androgen (~10-fold) circulating in males vs. females.

In our studies, we have found that AR overexpression in the mature osteoblast population *in vivo* results in a low turnover state with increased trabecular bone volume, but a significant reduction in cortical bone area due to inhibition of bone formation at the endosteal surface and a lack of marrow infilling. Every measure of biomechanical responsiveness is significantly inhibited. The most remarkable aspect of the phenotype is a dramatic reduction in osteoblast vigor. *Ex vivo* analysis of osteoblast differentiation in primary culture demonstrates that androgen directly inhibits proliferation, osteoblast differentiation, mineral accumulation and mineralized nodule formation. Data are also consistent with an inhibition of osteoclastogenesis, consistent with the low turnover phenotype. Thus, primary outcomes of analyses through year four demonstrate that enhanced androgen signaling in mature osteoblasts/osteocytes inhibits cortical bone formation, and results in changes that are detrimental to matrix quality, biomechanical competence and whole bone strength. The detrimental effects of androgen on bone were observed in both AR-transgenic lines. **This outcome was not predicted based on the literature available when these studies were initiated, and raise concerns regarding androgen administration during development or in eugonadal adults**.

Reductions in osteoblast vigor present *in vivo* likely reflect inhibition of osteoblast proliferation, differentiation and mineralization observed *in vitro* in mOB primary cultures and also in the signaling pathways identified in gene profiling analysis from whole bone by bioinformatic strategies. Since osteocytes are the most abundant cell type in bone, and AR levels are highest in osteocytes, these cells are likely a primary target for androgen signaling in bone. Gene expression profiling analysis suggests that osteocytes respond to chronic DHT treatment by modulation of signaling pathways that highlight the importance of

TGFß/BMP/Activin signaling, apoptosis/cell death, and modulation of signal transduction through membrane receptors, particularly Wnt pathways. The identification of important pathways may **provide insight into the development of therapeutics for treatment of low bone mass diseases, particularly to minimize inhibitory effects of direct androgen signaling on bone cells**.

Key Research Accomplishments
Accomplishments for 2007-2008 directly characterizing androgen action in the skeleton:

Peer reviewed publications:

- Semirale A. A., Wiren KM: Androgen administration has therapeutic advantages in the hypogonadal, but should be approached with caution in healthy adults. J Musculoskelet Neuronal Interact 2007; 7(4):361-367
- Wiren KM, Semirale A. A., Zhang X-W, Woo A, Tommasini SM, Price C, Schaffler M, Jepsen KJ: Targeting of androgen receptor in bone reveals a lack of androgen anabolic action and inhibition of osteogenesis. A model for compartment-specific androgen action in the skeleton. Bone 43:440–451, 2008

Chapters:

- Wiren KM: Androgens and Skeletal Biology: Basic Mechanisms. In: Osteoporosis, Third Edition (R Marcus, Feldman D, Nelson D, Rosen C, Eds), Academic Press, San Diego, CA, pp. 425-449*,* 2008
- Wiren KM: Androgen Action in Bone: Basic Cellular and Molecular Aspects. In: Osteoporosis: Pathophysiology and Clinical Management, Second Edition (R Adler, Ed), The Humana Press, Inc., Totowa, NJ, *in press,* 2008
- Wiren KM: Androgens: receptor expression and steroid action in bone. In: Principles of Bone Biology, Third edition (JP Bilezikian, LG Raisz and TJ Martin, Eds), Academic Press, San Diego, CA, pp 1001-1023, 2008

Abstracts:

- Zhang X-W, Wiren KM: Overexpression of androgen receptor in mature osteoblasts and osteocytes inhibits osteoblast differentiation. (Abstract #SU192) J Bone Miner Res, 2008
- Semirale A. A., Wiren KM: DHT Administration Is Effective for the Prevention of Hypogonadal Bone Loss. (Abstract #SU193) J Bone Miner Res, 2008

Reportable Outcomes:

One peer-reviewed publication (with one more in preparation) and two abstracts were published in 2007-2008 characterizing androgen action in the skeleton. In addition, a new subcontract has been established for the histomorphometric and µCT analysis for AR2.3- and AR3.6-transgenic mice in either the prevention or therapeutic androgen administration paradigm. In the work summarized here, we have completed the characterization of the second AR transgenic family set (AR2.3) with skeletally-targeted AR overexpression at 2 months of age. In contrast to the original AR3.6-transgenic family, the AR2.3-transgenic family demonstrates overexpression that is limited to mature osteoblasts and mineralizing osteocytes. Comparison of the phenotypes observed between the two lines has identified key target cells mediating specific effects of androgen on the skeleton, and demonstrates the envelope-specific effects of androgen signaling. Based on our extensive characterization of the consequences of androgen signaling, Dr. Wiren was invited to present this work at Osteomen 2008 in Santa Margherita, Italy. Currently our expression profiling and bioinformatic analyses is identifying signal

transduction pathways altered in endosteal samples from AR-transgenic cortical bone (from which the periosteum was stripped and marrow elements flushed) suggests that TGFß/BMP signaling is a major pathway influencing proliferation, osteoblast differentiation and mineralization. Data also suggests that IL-6 and NFkB signaling is altered to negatively influence osteoclastogenesis. Our *ex vivo* primary culture analyses support findings established in the *in vivo* models.

Conclusions:

These results have provided new insight into the importance of androgen action, through distinct AR transactivation, in mediating bone quality and changes that underlie envelope-specific responses. Our investigations of the mouse skeleton reveal that androgen signaling in immature osteoblasts and periosteal cells increases bone formation at the periosteal envelope (particularly in calvaria) and also influences processes that determine body composition and whole bone strength in AR3.6-transgenic mice. In contrast, analysis from AR2.3-transgenic mice suggests that signaling in mature osteoblasts/osteocytes primarily mediates the effects of androgens on matrix quality and/or mineralization with a negative impact on osteoblast vigor, and at least partially influences the effects of androgens to reduce bone turnover. A similar relationship may also exist in the human skeleton.

Thus, the effects of androgens are pervasive, influencing many organ systems in the body including bone. However, the mechanisms through which AR signaling influence osteoblast function remain controversial. We have shown previously that in mature osteoblasts, androgens inhibit osteogenesis and that AR signaling in vivo inhibits bone formation by differentiated osteoblasts and has detrimental consequences on matrix quality, bone fragility and whole bone strength (12, 13). We have also shown chronic DHT treatment inhibits both mitogenic signaling and MAP kinase activity in osteoblasts (24), and enhances osteoblast apoptosis (14). In order to evaluate cell autonomous affects of androgen on osteoblast function and identify important signal transduction pathways that are affected, mice with targeted AR overexpression were used as a source of primary osteoblasts. We show that androgen directly inhibits normal osteoblast proliferation, matrix production and mineralization. Overexpression of AR exacerbates the detrimental effects of DHT treatment. Consistent with the osteocyte as a target of androgen signaling, addition of DHT during the late stages of differentiation results in the largest decrease in marker protein levels. The results indicated that AR overexpression results in cell-autonomous inhibitory effects of androgen on proliferation and mineralization in vitro. These results raise concerns that with increasing androgen action (as for example associated with anabolic steroid abuse) in young animals with still-growing skeletons, the bone matrix material is likely to be more stiff, less ductile and more damageable, and suggest that young anabolic steroid abusers may be at higher risk of stress fractures. Importantly, supplemental levels of androgen in eugonadal adults are also detrimental. In contrast, analyses in the mature adult indicate that androgen may be useful to treat reductions in bone mineral in hypogonadal males.

In addition, androgen administration increases muscle mass, partially mediated by effects on stem cell lineage commitment (17), likely to indirectly influence bone density through biomechanical linkage. But as we have shown, improvement body composition after androgen replacement is observed only in hypogonadal males. At the same concentration and for the same duration, hypogonadal females do not demonstrate the same improvement with androgen replacement. Supplemental androgen in eugonadal adults tends to worsen, not improve, body compositional changes that occur with aging.

Some of the negative consequences of AR overexpression in mature osteoblasts we have observed in vivo are consistent with our *in vitro* analyses. For example, there are reports, some in clonal osteoblastic cell lines, of effects of gonadal androgen treatment on differentiation, matrix production and mineral accumulation mediated by AR signaling (18-20). These findings are variable however, with other reports of no effect or even inhibition of osteoblast markers (21-23), consistent with our gene expression analysis in AR-transgenic mice. In addition, the effect of androgens on osteoblast proliferation is controversial. We have previously demonstrated that either stimulation or inhibition of osteoblast viability by androgen can be observed, and these effects are dependent on the length of treatment. Transient administration of nonaromatizable DHT can enhance transcription factor activation and osteoblast proliferation, while chronic treatment inhibits both mitogenic signaling and MAP kinase activity (24). Chronic DHT treatment *in vitro* also results in enhanced osteoblast apoptosis (14). Thus, these in vitro reports are consistent with the detrimental changes in matrix quality and osteoblast vigor we observe in the AR-transgenic model *in vivo*.

In summary, one of the effects of enhanced androgen signaling is altered whole bone quality and susceptibility to damage in the young, which may be revealed under extreme physical conditioning such as that experienced during military training. Our data also indicate a concern with androgen supplementation in eugonadal adults, both male and female. Finally, it does appear that androgen may be effective in improving body composition, including increased lean and reduced fat mass, but only in hypogonadal males with reduced sensitivity to the improvement in females. Combined, these results support the central hypothesis of distinct androgen signaling throughout osteoblast differentiation. Androgen action in the skeleton is complex and will likely not provide for dramatically improved skeletal dynamics in the still-growing skeleton or the eugonadal adult, although the effect in the hypogonadal male is more positive. As noted above, **this outcome was not predicted based on the literature available when these studies were initiated, and raise concerns regarding androgen administration during development or in eugonadal adults**. It may be that the identification of important pathways can **provide insight into the development of therapeutics for treatment of low bone mass diseases, particularly to minimize inhibitory effects of direct androgen signaling on bone cells**.

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Targeting of androgen receptor in bone reveals a lack of androgen anabolic action and inhibition of osteogenesis
A model for compartment-specific androgen action in the skeleton

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ABSTRACT

Androgens are anabolic hormones that affect many tissues, including bone. However, an anabolic effect of androgen treatment on bone in eugonadal subjects has not been observed and clinical trials have been disappointing. The androgen receptor (AR) mediates biological responses to androgens. In bone tissue, both AR and the estrogen receptor (ER) are expressed. Since androgens can be converted into estrogen, the specific role of the AR in maintenance of skeletal homoeostasis remains controversial. The goal of this study was to use skeletally targeted overexpression of AR in differentiated osteoblasts as a means of elucidating the specific role(s) for AR transactivation in the mature bone compartment. Transgenic mice overexpressing AR under the control of the 2.3-kb α1(I)-collagen promoter fragment showed no difference in body composition, testosterone, or 17ß-estradiol levels. However, transgenic males have reduced serum osteocalcin, CTx and TRAPC5b levels, and a bone phenotype was observed. In cortical bone, high-resolution micro-computed tomography revealed no difference in periosteal perimeter but a significant reduction in cortical bone area due to an enlarged marrow cavity. Endocortical bone formation rate was also significantly inhibited. Biomechanical analyses showed decreased whole bone strength and quality, with significant reductions in all parameters tested. Trabecular morphology was altered, with increased bone volume comprised of more trabeculae that were closer together but not thicker. Expression of genes involved in bone formation and bone resorption was significantly reduced. The consequences of androgen action are compartment-specific; anabolic effects are exhibited exclusively at periosteal surfaces, but in mature osteoblasts androgens inhibited osteogenesis with detrimental effects on matrix quality, bone fragility and whole bone strength. Thus, the present data demonstrate that enhanced androgen signaling targeted to bone results in low bone turnover and inhibition of bone formation by differentiated osteoblasts. These results indicate that direct androgen action in mature osteoblasts is not anabolic, and raise concerns regarding anabolic steroid abuse in the developing skeleton or high-dose treatment in eugonadal adults.

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Introduction

Androgens are steroids that are generally characterized as anabolic hormones, with effects on many tissues including the brain, the immune system, the cardiovasculature, muscle, adipose tissue, liver and bone. Given the large increase in drug sales for testosterone (the major androgen metabolite), estimated at over 500% in the last fifteen years, analysis of the biological consequences of androgen signaling should receive considerable research attention. However, the specific effects of androgen on the skeleton remain poorly characterized and understudied. Since osteoporosis is often coupled with a hypogonadal state in both men and women, sex steroids are implicated in the maintenance of skeletal health. Although both estrogen and androgen circulate in men and women, albeit at different levels, the influence of each on the remodeling skeleton is distinct [34,55]. Consistent with this, combination therapy with both estrogen and androgen provides an improved response in post-menopausal women compared to estrogen alone [2,47]. Estrogens are thought to act to maintain adult bone mass predominantly through an inhibition of bone resorption by the osteoclast, *i.e.* as anti-resorptive agents, which protects the skeleton from further loss of bone. Non-aromatizable androgens such as 5α-dihydrotestosterone (DHT), on the other hand, have been proposed as possible bone anabolic agents that increase bone formation and bone mass [30,35].

In support of an anabolic effect of androgen on the skeleton, free testosterone concentrations have been shown to correlate with bone mineral density (BMD) in elderly men [56], however testosterone levels

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also correlated with muscle mass and strength. Testosterone treatment is effective at ameliorating bone loss during aging, but only in men with low testosterone levels [8,61]. Conversely, men undergoing androgen deprivation therapy for prostate cancer show significantly decreased BMD [49] and an increase in clinical fractures [32]. During growth, there are gender differences in skeletal morphology that develop with puberty particularly in cortical bone, with radial expansion that is predominantly observed in boys [31]. Combined, these findings suggest that androgens serve important functions to both maintain bone mass in the adult and to influence the growing, modeling skeleton (see[63]).

Nevertheless, a controversy exists regarding the consequences and/or importance of androgen signaling on skeletal homeostasis. Whether the observed effects of circulating testosterone are due to direct effects on bone is complicated by the fact that androgens influence a variety of tissues known to be associated with bone health, most importantly muscle. Nonetheless, bone is a direct target tissue with respect to androgen action. AR is expressed in the cell types required for skeletal formation and homeostasis, including mesenchymal stromal precursors [4], osteoblasts [1], osteocytes [1,64] and osteoclasts [57]. An additional complication for interpretation of the direct effects of testosterone results from the consequences of its metabolism. Since testosterone serves as the substrate for estradiol synthesis through the action of the enzyme aromatase, systemic testosterone may have effects mediated predominantly or exclusively through activation of estrogen receptor (ER) signaling. Therefore, a specific role for AR signaling cannot be inferred with simple testosterone therapy.

In addition, not all of the studies examining the association of testosterone levels with BMD in adults have actually shown a positive correlation. In general, correlations between bone mass and serum androgen concentrations in adult men have been either weak or insignificant [17,42,48]. Furthermore, many of the various clinical trials examining androgen therapy have been unable to demonstrate robust effects on bone mass, including treatment with anabolic steroids [11]. In most studies that do show an increase in BMD, the most marked improvement is observed only in men with the lowest testosterone levels [61]. Notably, an anabolic effect of androgen treatment on bone in eugonadal men (or in women) has not been observed, in contrast to known anabolic dose-dependent effects to increase muscle mass [5]. For these reasons and because of concerns about safety, androgen replacement even in hypogonadal men remains a controversial issue [20]. Given the modest therapeutic benefit observed with androgen therapy [33], speculation has arisen that a portion of the positive effect of androgens on bone mass may be mediated indirectly through known effects to increase muscle mass and strength. An increase in lean mass would have beneficial effects on BMD though biomechanical linkage and skeletal adaptation. Consistent with this suggestion, Murphy et al [39] have shown that administration of the synthetic anabolic androgen oxandrolone to severely burned children increases lean body mass three to six months before an increase in bone mineral content is observed.

Not surprisingly given the complex nature of bone tissue, systemic androgen administration has shown distinct responses in different skeletal compartments, *i.e.*, cortical, trabecular (cancellous) or intramembranous bone. In hypogonadal settings, a beneficial response to androgen therapy is observed in the trabecular compartment, the more active surface in bone, with an increase in bone mass. However, this relative increase in bone mass occurs with suppression of bone resorption, with micro-architectural changes demonstrating an increase in trabecular number but not thickness. For example, histomorphometric analysis of androgen replacement in hypogonadal male mice has shown that AR activation preserves the number of trabeculae but does not maintain thickness, volumetric density or mechanical strength [38]. Notably, these studies also demonstrated that the bone-sparing effect of AR activation is distinct from the bone-sparing effect of ERα. In addition, androgen appears to play an important role in intramembranous bone formation [16]. Finally, there are reports of increased cortical bone mass, as a consequence of increased bone width and surface periosteal expansion (see[59,63]). High-dose testosterone therapy over 2 years in (genetic female) female-to-male transsexuals resulted in increased areal BMD at the femoral neck, in a setting where estradiol declined to post-menopausal levels [54]. In men with constitutional delay of puberty, impaired periosteal expansion is observed [68]. Taken together, these reports indicate that androgens *in vivo* act to maintain trabecular bone mass through inhibition of osteoclast activity and to expand cortical bone at the periosteal surface. While these findings argue that androgen positively affects cortical bone at the periosteal surface (see[67]), what is lacking is clear documentation of an anabolic effect to increase bone formation in mature osteoblasts and osteocytes. Thus, the direct consequences of androgen action on differentiated osteoblasts *in vivo* remain unclear, and mechanisms underlying potential positive outcomes on bone formation and bone mass remain uncharacterized.

Concentrations of estrogen and androgen receptors vary during osteoblast differentiation, with AR levels highest in mature osteoblasts and osteocytes [64]. Since osteocytes are the most abundant cell type in bone [50], these cells are likely an important target cell for androgen action, and may represent a central mediator for skeletal responses to testosterone therapy *in vivo*. The goal of this study was to use skeletally targeted overexpression of AR as a means of elucidating the specific role(s) for AR transactivation in the mature differentiated osteoblast. AR overexpression was targeted by the col2.3 promoter and was chosen for several reasons: the skeletal expression patterns for this promoter are bone-selective and well-characterized (see[24–26,36,37]); with strong col2.3 promoter activity in differentiated osteoblasts/osteocytes and mineralizing nodules [25,26,36] but not in osteoclasts [7]. Promoter activity varies in different bone compartments. In intramembraneous bone, strong expression was seen in cells at osteogenic fronts of parietal bones but not in the suture [36]. In long bones, strong transgene expression was observed in most osteoblasts on endocortical surfaces, and in a large proportion of osteocytes in femurs throughout cortical bone but with no expression in periosteal fibroblasts [25,26]. In the trabecular area of metaphyseal bone, strong expression was observed at all developmental stages [26].

A distinct advantage of employing a transgenic model is the enhancement of androgen signaling in a specific target *in vivo*, *e.g.*, mature osteoblasts and osteocytes, as a consequence of increased AR abundance. The AR2.3-transgenic model allows for characterization of skeletal responses in the absence of changes in circulating hormone (testosterone or 17b-estradiol) that occurs with global genetic manipulations, to take advantage of increased sensitivity to androgen in distinct skeletal sites for analysis of compartment-specific effects. At the same time, manipulation of androgen action through AR overexpression, rather than systemic administration, gonadectomy or global knockout, excludes effects that would occur at other androgen target tissues *in vivo* including muscle and fat. Here we describe the skeletal consequences of enhanced androgen signaling that is restricted to mature osteoblasts and osteocytes, employing the 2.3-kb type I collagen promoter to control AR overexpression.

Materials and methods

Cloning of expression plasmids

The pBR327-based plasmid col2.3-ßgal-ClaPa contains the basic rat collagen Iα1 promoter sequence − 2293 to + 115 (provided by Dr. David Rowe, University of Connecticut Health Center), which served as the starting vector. BamHI sites were added to the rat AR cDNA (provided by Dr. Shutsung Liao, University of Chicago) with PCR primers. The PCR product was T/A cloned in pCR 2.1-TOPO vector (Invitrogen Corp., Carlsbad, CA, USA). Finally the BamHI-rAR fragment was cloned into the BamHI site in the col2.3-ßgal-ClaPa (after removal of the ßgal cDNA sequences), to give the expression construct referred to as the AR2.3-transgene. The correct sequence and orientation of the AR insert was verified by direct DNA sequencing.

Generation of AR2.3-transgenic mice

AR2.3-transgenic mice were produced using standard technology by the Oregon Health and Science University (OHSU) Transgenic Mouse Facility, following methodology previously described [67]. Embryos were obtained from matings of C57BL/6 males × DBA/2J

females (B6D2F1). Founder mice were identified by PCR genotyping and mated with B6D2F1 (Jackson Labs, Bar Harbor, ME, USA) to produce F2 litters. Transgenic mice were healthy and transmitted the transgene at the expected frequency. The generation and use of transgenic mice were performed according to institutional, local, state, federal and NIH guidelines for the use of animals in research under an Institutional Animal Use and Care Committee-approved protocol.

Animals

AR2.3-transgenic mice were bred to B6D2F1 mice (Jackson Labs); both genders were employed. The mice had free access to tap water and were fed a diet containing 1.14% calcium, 0.8% phosphorous, 2200 IU/kg vitamin D3, 6.2% fat and 25% protein (Purina PMI Nutrition International, St. Louis, MO, USA). All animals were weighed weekly, and body length (nose to rump) was determined at monthly intervals over six months (n=4–5). For analysis, the animals were sacrificed under CO_2 narcosis by decapitation. The right femur was used for measurement of cortical and trabecular volumetric density and geometry by *ex vivo* micro-computed tomography (μCT), followed by destructive analysis of whole bone biomechanical properties. The left femur was used for dynamic histomorphometric analysis. The length of the femur was measured from the femoral head to the distal condyles. In addition, a variety of tissues/organs were collected for RNA isolation or histological and immunohistochemical analyses. For RNA isolation, tibia was cleaned of muscle tissue and aseptically dissected. After removal of the epiphyseal area, marrow was briefly flushed with sterile phosphate buffered saline and the bone was frozen in liquid nitrogen and stored at −80 °C until RNA isolation as described below.

Primary calvarial osteoblast culture and western blot analysis

Primary osteoblastic cells were isolated after collagenase digestion from fetal calvaria from both wild-type and AR2.3-transgenic fetal mice. Calvariae were isolated from 3–6 day old mice after genotyping and subjected to four sequential 15-min digestions in a mixture containing 0.05% trypsin and 0.1% collagenase-P at 37 °C. Cell fractions 2–4 were pooled and plated at 8000 cells/cm^2 in MEM supplemented with 10% FBS. Beginning at confluence around day 7, cultures were switched to differentiation medium in phenol-red free BGJb (Fitton-Jackson modification) containing 50 μg/ml ascorbic acid. From day 14 on, 5 mM ß-glycerophosphate was added to the differentiation media. Whole cell lysates were prepared with lysis buffer. Equal amounts of cell extract were electrophoresed on a 10% SDS-polyacrylamide gel, and the separated proteins were transferred to an Immobilon-P polyvinylidene-difluoride transfer membrane (Millipore, Bedford, MA, USA). AR abundance was determined by immunoblotting with polyclonal rabbit AR antibodies (ARN-20) purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and used at 1:300 dilution. The α-tubulin antibody (T9026) was a mouse monoclonal antibody purchased from Sigma and was used at 1:1000 dilution. Bound antibodies were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA) on Kodak X-AR5 autoradiographic film. The analysis of α-tubulin was used for protein loading control.

Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted and the concentration was measured at 260 nm using a spectrophotometer, with purity assessed by the $A_{260/280}$ ratio. RNA integrity was confirmed on a 1% agarose gel after SYBR Gold staining (Invitrogen Corp), and qRT-PCR was performed as previously described [19]. Intron-spanning primers for tibial RNA analysis were purchased pre-designed from Qiagen (Valencia, CA, USA). Transgene-specific RT primers were forward 5′-GCATGAGCCGAAGCTAAC-3′ and reverse 5′-GAACGCTCCTCGATAGGTCTTG-3′ designed using Oligo Software (Molecular Biology Insights, Inc. Cascade CO, USA), and specifically amplified colAR using sites in the collagen untranslated region and AR near to those used for genotyping. Fold regulation was determined by normalizing all values to the mean of the value in calvaria.

Serum biochemistry and hormone analysis

Serum specimens from 2-month-old female and male mice of both genotypes were collected and stored at −20 °C until analysis was performed (n=6–17). Blood samples were obtained under anesthesia by cardiac puncture. Serum analysis was as previously described [67], with 17ß-estradiol measured by radioimmunoassay (RIA) using Immuchem Double Antibody 17ß-Estradiol RIA (ICN Biomedicals Inc., Costa Mesa, CA, USA); testosterone measured by enzyme linked immunoassay from Diagnostic Automation Inc. (Calabasas, CA, USA); osteocalcin quantitated by ELISA (Biomedical Technologies Inc., Stoughton, MA, USA); and OPG determined by immunoassay kit (R&D systems, Minneapolis, MN, USA). Indices of bone resorption *in vivo* were quantitated using the serum biochemical marker C-terminal telopeptide of collagen (CTx) analyzed by a RatLaps ELISA kit (Nordic Bioscience Diagnostics A/S, Herlev, Denmark). Assays were performed using 20 μl serum aliquots in duplicate following the manufacture's recommendations. Intraassay variation was 5.6%, and interassay variation was 10.5%. Serum TRAPC5b activity was determined using MouseBoneTRAP Assay (Immunodiagnostic Systems Inc., Fountain Hills, AZ, USA) using 25 μl serum aliquots in duplicate following the manufacturer's protocol.

Histochemical analysis of calvaria

Immunohistochemical analysis was performed on representative calvaria from 2-month-old mice. Calvaria were fixed, decalcificied in Immunocal (Decal Corp., Tallman, NY, USA), and sections were processed by dehydration, paraffin infiltration and embedding. Tissue sections (5–6 μm) were cut, processed and subjected to immunohistochemical staining after incubation in a primary antibody directed against AR N-terminus (ab3510, 4 μg/ml; Abcam Inc, Cambridge, MA, USA) at 4 °C overnight. For AR detection, sections were incubated for 1 h in a biotinylated goat antirabbit secondary antibody (1:200; Vector Laboratories, Burlingame, CA, USA). Following rinses, sections were incubated for 60 min in avidin-biotin complex (1: 1000; Vectastain Elite; Vector Laboratories). After 30 min of rinsing, sections were incubated for 10 min with a diaminobenzidine (DAB) solution (0.05% DAB) activated by 0.001% hydrogen peroxide. Slides were counterstained with hematoxylin.

Dynamic histomorphometry

Bone formation and resorption during the last week of growth was assessed by dynamic histomorphometric measures after fluorochrome labeling (n=8–20 males; 10–15 females). Prior to sacrifice, 2-month-old mice received two fluorochrome labels by intraperitoneal (ip) injection [oxytetracycline hydrochloride (Sigma, St. Louis, MO, USA) at 30 mg/kg and calcein green (Sigma) at 10 mg/kg], given 10 days and 3 days before death, respectively. Left femora were dissected and processed non-decalcified for plastic embedding as previously described [23]. Cross-sections (100 μm) through mid-diaphysis were prepared using a diamond-wafering saw (Buhler, Lake Bluff, IL, USA), then polished to a thickness of 30 μm. Sections were left unstained and dynamic histomorphometry was carried out using a light/epifluorescent microscope and a semiautomatic image analysis system (OsteoMetrics, Inc., Decatur, GA USA). Standard measures of bone formation and resorption were determined for both the periosteum and endocortex, including mineral apposition rate (MAR, μm/day), mineralizing perimeter ([dL.Pm+sL.Pm/2]/B.Pm, %), bone formation rate (BRF/B.Pm, μm^2/μm/day), and eroded perimeter (%). Bone formation rate (perimeter referent) was calculated by multiplying mineralizing perimeter by mineral apposition rate. In addition, the cross-sectional area and cortical area were measured on cortical cross-sections and cortical thickness calculated. The terminology and units used were those recommended by the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research [44].

Bone morphology and microstructure

The morphological consequences of increased AR expression in osteoblastic/osteocytic cells in AR2.3-transgenic animals were evaluated in 2-month-old male and female mice by dual energy X-ray absorptiometry (DXA) and quantitative μCT. Areal BMD, bone mineral content (BMC) and body composition was measured by whole body DXA using a mouse densitometer (PIXImus2, Lunar, Madison WI, USA). Right femurs from each genotype (n=10–21 males; 13–19 females) were examined for diaphyseal cross-sectional morphology and tissue mineral density (TMD) using an eXplore Locus SP Pre-Clinical Specimen MicroComputed Tomography system (GE Medical Systems, London, Ontario) as described previously [22]. Area measures were body weight adjusted to reduce variability. Three-dimensional images of the entire femur were obtained at an 8.7 μm voxel size and individually thresholded using a standard segmentation algorithm [43]. A 3 mm region of the reconstructed mid-diaphysis, corresponding to the typical failure region for 4-point bending (see below), was examined. Determination of cross-sectional morphology was performed using custom analysis program (MathWorks, v. 6.5; The MathWorks, Inc., Natick MA, USA) [22]. Trabecular morphometry of the distal metaphysis, including bone volume fraction (BV/TV) and trabecular thickness (Tb.Th), trabecular number (Tb.N) and trabecular separation (Tb.Sp), was assessed using Microview Advanced Bone Analysis (GE Medical Systems, v. 1.23).

Images from the μCT analysis were also used to quantify TMD as described previously [22]. TMD is the average mineral value of the bone voxels alone, expressed in hydroxyapatite (HA) density equivalents. TMD was calculated by converting the grayscale output of bone voxels in Hounsfield units (HU) to mineral values (mg/cc of HA) through the use of a calibration phantom hydroxyapatite (SB3: Gamex RMI, Middleton, WI, USA) [TMD=average bone voxel HU/average HA phantom HU*1130 mg/cm^3 (HA physical density)] [22]. The same calibration phantom was used for all scans to normalize mineral density measurements and to account for possible variability among scan sessions.

Mechanical testing

Following DXA and μCT analysis, the right femurs were subjected to destructive testing to establish whole bone mechanical properties. Femurs were loaded to failure in 4-point bending. All whole bone-bending tests were conducted by loading the femurs in the posterior to anterior direction. The load-deflection curves were analyzed for stiffness (the slope of the initial portion of the curve), maximum load, post-yield deflection (PYD), and work-to-failure as described previously [23]. Stiffness and maximum load were adjusted for body weight.

Statistical analysis

All data were analyzed using Prism software v4 (GraphPad Software, Inc., San Diego, CA, USA). Significance of difference between wild-type and AR2.3-transgenic mice was assessed by an unpaired two-tailed t-test using Welch's correction. Body lengths and weights were analyzed by repeated measures two-way ANOVA for the effects of gender and genotype. All data are shown as mean±standard error of the mean (SEM).

Results

Generation of transgenic mice with enhanced androgen signaling in mature osteoblasts and osteocytes

Confusion exists regarding the *in vivo* action of androgens in bone due to metabolism to estrogen, because androgen influences many tissues in the body, and many months of treatment are required to observe improvement in BMD. The AR2.3-transgenic animal model was created to determine the specific physiologic relevance of androgen action in the mature osteoblast/osteocyte population in bone, through tissue-specific overexpression of AR. This line is distinct from our previously described AR3.6-transgenic model, with AR overexpression in stromal precursors, periosteal fibroblasts and throughout the osteoblast lineage directed by the col3.6 promoter [67]. A transgene cassette (AR2.3) was cloned as described in Materials and methods and AR2.3-transgenic mice were created following standard procedures. Positive founders were identified by PCR genotyping and were bred to wild-type B6D2F1 mice; two AR2.3-transgenic lines (lines 219 and 223) derived from independent founders have been retained. Southern analysis confirmed a single insertion site for the AR2.3-transgene (data not shown). Table 1 lists the qRT-PCR analysis of expression of the AR2.3-transgene in various tissues, showing the expected bone targeting with highest levels in calvaria but ~100–3000-fold lower levels in muscle, skin, heart, intestine, kidney, liver, lung and spleen.

Phenotype in AR2.3-transgenic mice with bone-targeted AR overexpression

To begin to characterize the phenotype of AR2.3-transgenic mice, we first determined body weight gain and nose–rump length over a 6-month period. At birth, animals were indistinguishable and as the mice aged, AR2.3-transgenic males and females gained length and weight similar to wild-type controls (Figs. 1a, b). Body composition and bone density were evaluated by DXA at 2 months in male and female AR2.3-transgenic mice and wild-type littermate controls. While systemic androgen treatment is known to affect body compositional changes, no difference was noted in either lean mass (Fig. 1c) or fat mass (Fig. 1d) in either males or females, consistent with skeletal targeting of the AR transgene. In addition, areal BMD (Fig. 1e) and BMC (Fig. 1f) were not significantly different in males or females, indicating a lack of effect on periosteal surfaces as expected.

AR2.3-transgenic mice were next evaluated for serum biochemistry and hormone levels at 2 months of age. As expected with bone targeting of AR overexpression, serum testosterone and estradiol levels were not significantly different between littermate controls and transgenic animals in either sex (Figs. 2a, b). There were also no significant differences in serum calcium levels between transgenic mice and littermate controls (Fig. 2c). Interestingly, there was a significant ~50% decrease in serum osteocalcin levels in male AR2.3-transgenic animals ($P<0.01$, Fig. 2d), but not in females. Serum OPG showed little difference between genotypes (Fig. 2e), but bone resorption markers TRAPC5b and CTx (that mostly reflect cortical bone properties) were significantly reduced in male AR2.3-transgenic mice ($P<0.05$, Figs. 2f, g).

To further evaluate the phenotype of AR2.3-transgenic mice, we characterized AR protein expression *in vivo* by immunohistochemical analysis in calvarial sections from both sexes (Fig. 2h). The antibody used does not distinguish between endogenous AR and the product of the AR2.3-transgene, so immunostaining represents combined levels for both proteins. AR expression is seen in osteocytes in wild-type mice (brown color combined with blue color from hematoxylin counterstaining), in sections from the middle of the calvaria (bottom panels for each sex). Consistent with increased AR2.3-transgene expression, robust AR expression is seen in osteocytes in both male and female transgenic mice (bottom panels for each sex). AR is also seen in differentiating osteoblasts at the innermost layer at the bone surface (upper panels for each sex). Thus, increased AR expression in osteocytes and the most differentiated osteoblasts is seen in both male and female transgenic animals, consistent with previous characterization of promoter activity [25,26]. There was no notable difference between the sexes or between independent families (data not shown). Morphological changes were evaluated by H&E staining (data not shown); there was no difference in calvarial width between wild-type and AR2.3-transgenic mice of either gender, nor between the independent AR2.3-transgenic families 219 and 223. In contrast, calvaria from male AR3.6-transgenic mice, with AR overexpression in periosteal fibroblasts and throughout the lineage, demonstrated substantial calvarial thickening [67]. Finally, we examined AR levels by Western analysis in *ex vivo* studies using primary calvarial osteoblast cultures derived from wild-type *vs.* transgenic mice. As shown in Fig. 2i, increased AR levels are seen in mature osteoblasts/osteocytes at day 30 in differentiating cultures from transgenic mice.

Table 1
Analysis of transgene expression in a variety of tissues from AR2.3-transgenic mice

Tissue	AR2.3-tg level	Fold difference
Calvaria	1.0000±0.2373	n.a.
Thymus	0.0066±0.0011	−152
Lung	0.0054±0.0005	−185
Heart	0.0047±0.0019	−213
Kidney	0.0044±0.0006	−227
Fat	0.0027±0.0007	−370
Spleen	0.0025±0.0004	−400
Muscle	0.0006±0.0000	−1667
Skin	0.0006±0.0002	−1667
Ear	0.0004±0.0001	−2500
Liver	0.0004±0.0002	−2500
Tendon	0.0003±0.0000	−3333
Intestine	0.0000±0.0000	n.d.

Tissues listed were harvested from male AR2.3-transgenic mice and total RNA was isolated ($n=5$). Expression of the transgene was evaluated by real-time qRT-PCR analysis after normalization to the total RNA concentration using RiboGreen [19]. Data are expressed relative to the expression level in calvaria as mean±SEM. n.a., not applicable; n.d., not detectable.

Enhanced androgen signaling results in altered bone morphology and reduced cortical area in male transgenic mice

Overall cortical bone morphology and femoral structure were quantified from high-resolution μCT images. Measures obtained for morphological assessment from μCT analysis are described in Fig. 3a. No effect of AR2.3-transgene expression on total cross-sectional area or surface periosteal perimeter was observed in either males or females (Figs. 3b, e). However, marrow cavity area was significantly increased in transgenic males (*i.e.*, reduced infilling occurred; $P<0.01$, Fig. 3c). Given no compensatory changes in the periosteal layer, this inhibition resulted in a modest but significant reduction in cortical bone area in male transgenic mice ($P<0.05$, Fig. 3d). Thus, enhanced AR signaling in mature osteoblasts has significant inhibitory effects on overall femoral cortical bone area. This morphological difference at the diaphysis was not observed in female transgenic mice. We also evaluated polar moment of inertia and tissue mineral density (TMD) at the mid-diaphysis. While there was no significant effect on polar moment of inertia (Fig. 3f), transgenic males show a significant reduction in TMD ($P<0.001$; Fig. 3g).

Reduced endocortical bone formation with bone-targeted AR overexpression

Because of the changes observed with the μCT analysis in cortical bone area in transgenic males, dynamic histomorphometric analysis was carried out at the femoral diaphysis using fluorescent imaging. Fluorochromes were administered (oxytetracycline followed by calcein) to label new mineral deposition. Fig. 3h shows patterns of bone formation in images of fluorochrome labeling from femoral cross-sections. The AR2.3-transgenic males (upper panel) show a

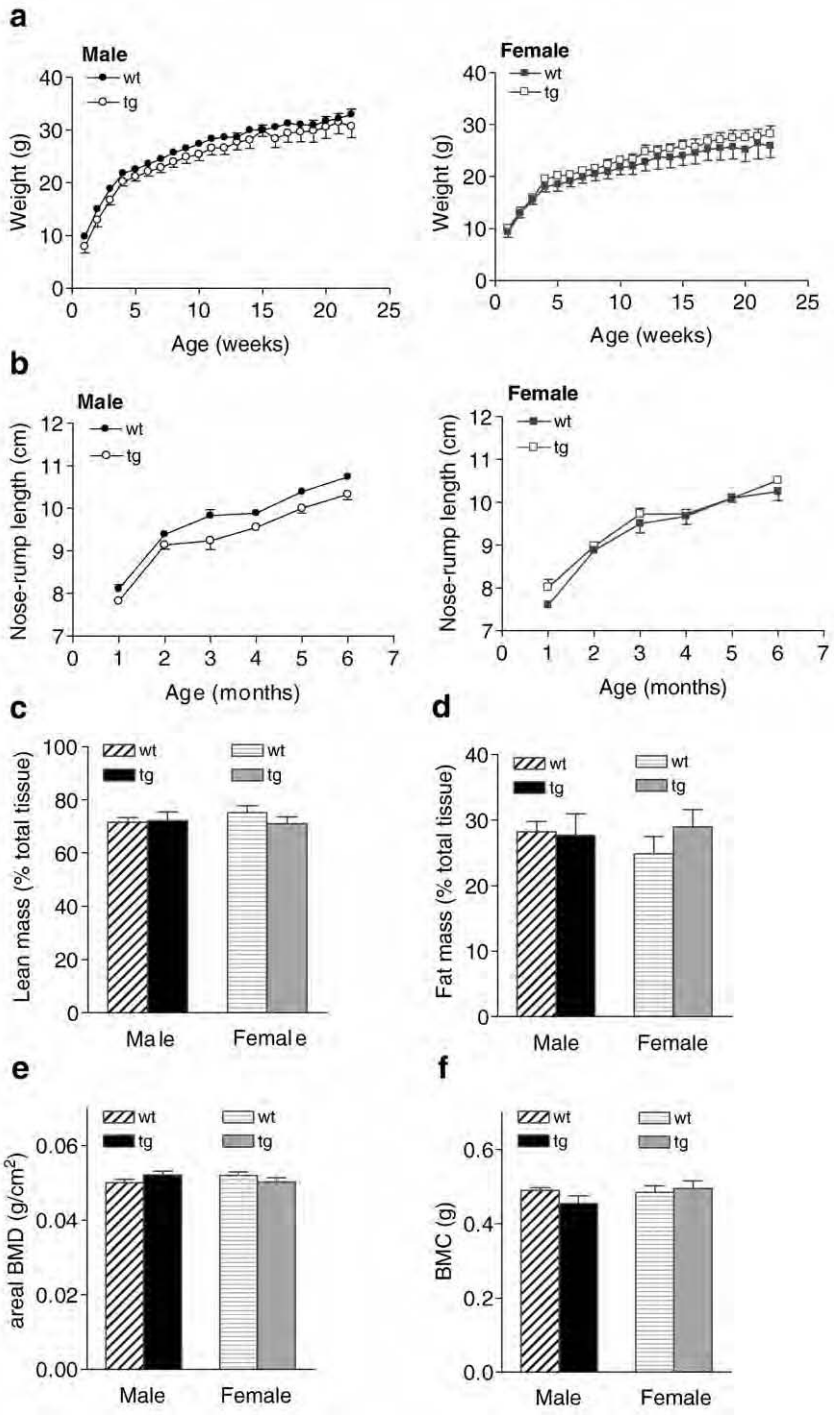

Fig. 1. Weight changes and body composition analysis in AR2.3-transgenic mice. Body weight and nose–rump-length determinations were carried out weekly or monthly, respectively, over six months in both genders in both wild-type (wt) and AR2.3-transgenic (AR2.3-tg) mice. a. Weight gain in growing male (left) and female (right) mice. Analysis for the effects of time and genotype by repeated measures two-way ANOVA in males revealed an extremely significant effect of time (F=218.36; P<0.0001) but not genotype, and with no interaction; females were similar (F=114.80; P<0.0001). b. Nose–rump length in male (left) and female (right) mice. Analysis by repeated measures in males revealed an extremely significant effect of time (F=228.54; P<0.0001) and an effect of genotype (F=15.87; P<0.01) with no interaction; females only showed an effect of time (F=149.48; P<0.0001). Data is shown as mean±SEM, n=4–5. DXA was performed on 6-month-old AR2.3-transgenic and littermate control mice to assess bone mineral, lean mass and fat mass. c. Lean mass adjusted for total tissue mass. d. Fat mass adjusted for total tissue mass. e. Areal BMD (minus head). f. BMC. Values are expressed as mean±SEM, n=4–10.

dramatic lack of labeling at the endocortical surface compared with wild-type controls (lower panel). Consistent with these fluorescent images, quantitative dynamic histomorphometric analysis revealed inhibitory responses at the endocortex in transgenic males, with strong inhibition (~70%) of BFR/B.Pm (P<0.001; Fig. 3i) and ~50% reduction in MAR, the latter being a measure of osteoblast vigor (P<0.001; Fig. 3j). Eroded perimeter was reduced at the periosteum (P<0.001; Fig. 3k), while mineralizing perimeter (M.Pm/B.Pm) showed inhibition at the endocortex (P<0.01) but stimulation at the periosteum (P<0.01) in Fig. 3l. The modest increase in periosteal activity seen does not parallel a change in cortical bone morphology characterized by μCT analysis, likely since the labeling is representative of mineralization patterns only for the period of time that the labels are present.

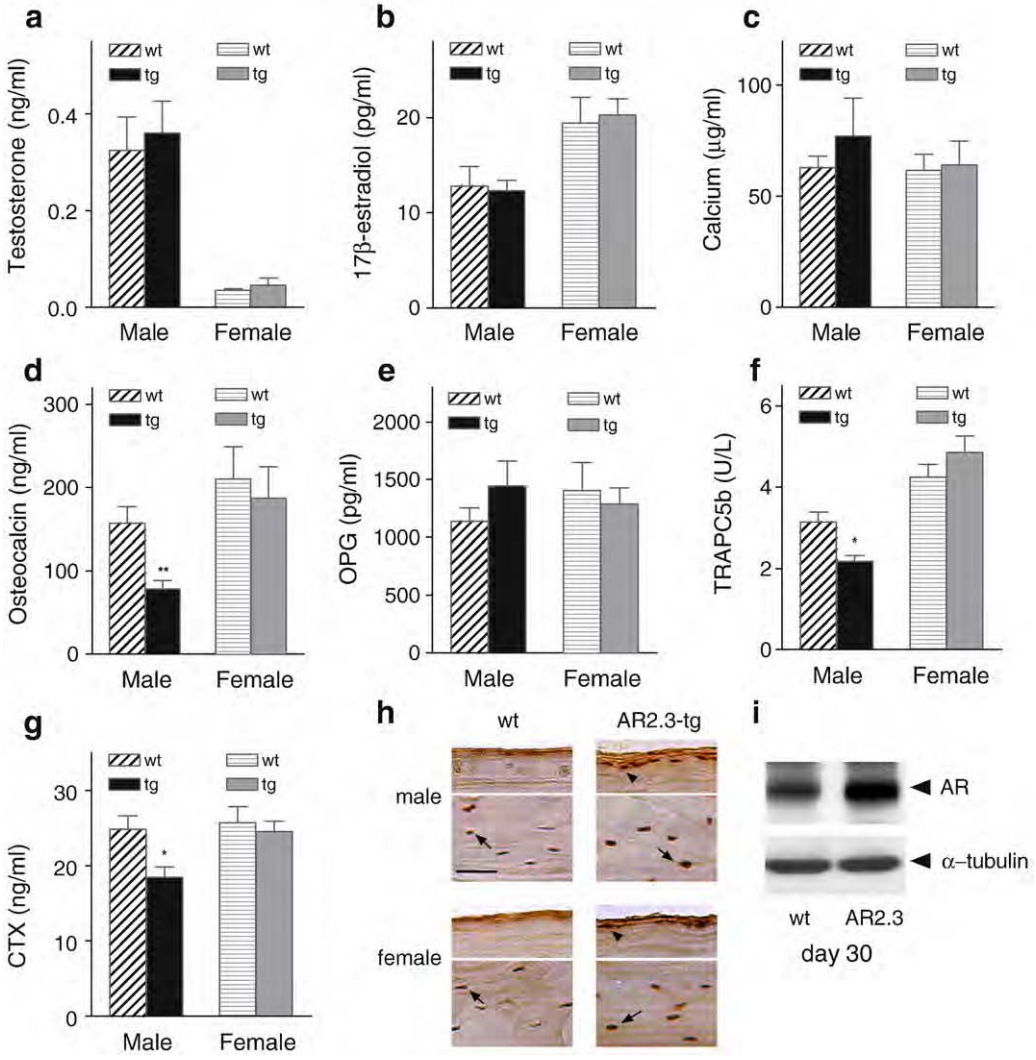

Fig. 2. Phenotypic characterization of serum markers and AR overexpression in AR2.3-transgenic animals. Comparisons were performed between wild-type littermate control (wt) and AR2.3-transgenic (AR2.3-tg) animals. Serum from 2-month-old mice was analyzed to determine levels of hormones and markers of calcium metabolism. Assays were performed in duplicate by RIA for 17ß-estradiol or EIA for testosterone, OPG and intact mouse osteocalcin, and for calcium by the colorimetric cresolphthalein-binding method. a. Testosterone. b. 17ß-estradiol. c. Calcium. There were no statistical differences between the genotypes for 17ß-estradiol, testosterone or calcium levels. Values are expressed as mean ± SEM, $n = 6$–17. d. Osteocalcin. e. OPG. f. TRAPC5b. g. CTx. Osteocalcin, TRAPC5b and CTx were all significantly reduced in male AR2.3-transgenic mice. Values are expressed as mean ± SEM, $n = 3$–8. *$P < 0.05$; **$P < 0.01$ (*vs.* gender-appropriate wild-type control). h. Immunohistochemical analysis of AR levels in calvaria isolated from 2-month-old mice. Sections were subjected to immunohistochemical staining after demineralization and paraffin embedding. For each sex, the top panel represents a section at the calvarial surface to focus on osteoblasts (arrowheads) while the bottom panel represents a section through the center of the bone to show osteocytes (arrow). Representative sections are shown. AR abundance was visualized with rabbit polyclonal antisera for male and female mice from wt and AR2.3-tg mice. AR is brown and the nucleus is purple after DAB incubation and counterstaining with hematoxylin. Scale bar = 50 μm. i. AR levels by Western blot analysis during *ex vivo* differentiation of calvarial osteoblasts derived by collagenase digestion from wild-type or AR2.3-transgenic mice. Analysis was performed at day 30 in mineralizing cultures. Control for loading was characterized by α-tubulin levels.

Ability to resist fracture is impaired in male AR2.3-transgenic mice

To analyze whole bone biomechanical and failure properties, femurs from 2-month-old wild-type and AR2.3-transgenic animals were loaded to failure in 4-point bending at 0.05 mm/s. Although overall geometry of the femur showed no obvious differences between wild-type and transgenic mice (Fig. 4a), failure properties were significantly impaired. Diaphyseal strength assessed as both maximum load ($P < 0.05$, Fig. 4b) and stiffness ($P < 0.05$, Fig. 4c) was decreased slightly by about 10% in male transgenics (with no change in females), consistent with the decreased cortical bone area (see Fig. 3c). However, a dramatic impairment was seen in male transgenic bones in their ability to resist fracture. They were more brittle (less ductile), with an approximately 40% decrease in post-yield deflection ($P < 0.05$, Fig. 4d). Work-to-failure was reduced by nearly 30% compared to wild-type control bones ($P < 0.05$, Fig. 4e). Male AR2.3-transgenic mice in this cohort showed no difference in femoral length or weight (Figs. 4f, g).

AR overexpression in bone results in increased trabecular bone volume in male transgenic mice

We also used μCT analysis to evaluate the consequences of AR overexpression on the trabecular bone compartment. Visualization of trabecular bone in the metaphysis after manual subtraction of the cortical shell shows an increase in trabecular bone volume in male AR2.3-transgenic mice (Fig. 5a). To better characterize trabecular micro-anatomy and architecture, static histomorphometric analysis was performed from images of the metaphyseal trabecular region (Figs. 5b–e). Male AR2.3-transgenic mice showed an ~35% increase in trabecular bone volume as a percent of tissue volume (BV/TV; $P < 0.05$; Fig. 5b), consistent with the μCT image. The increase in trabecular

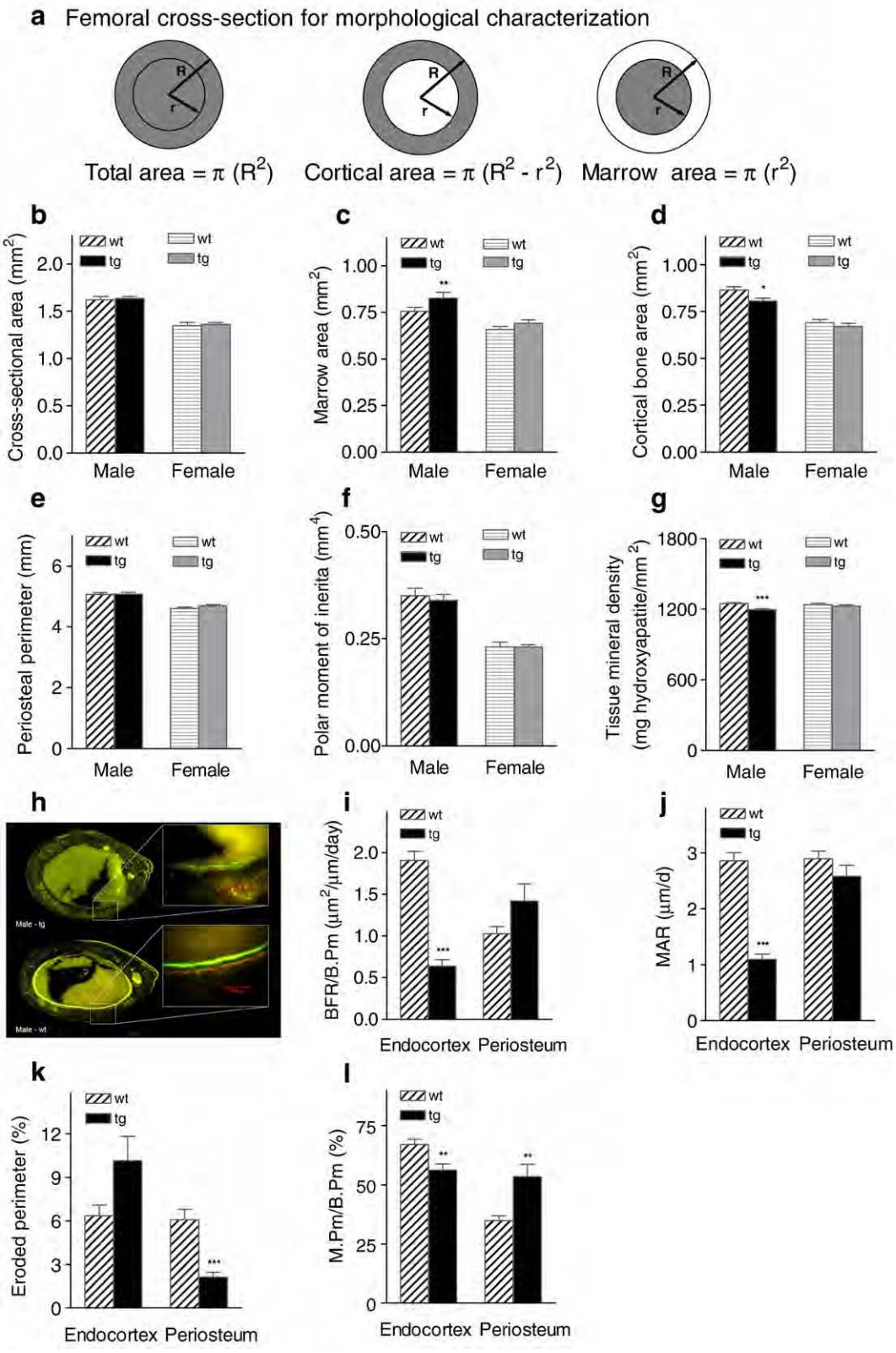

Fig. 3. Cortical morphology, structural analysis and bone formation rates in AR2.3-transgenic mice. Femurs were isolated from 2-month-old male and female wild-type (wt) or AR2.3-transgenic mice (tg) and subjected to high-resolution μCT imaging at mid-diaphysis. a. Parameters for morphological characterization by μCT. b. Total cross-sectional area. c. Marrow cavity area. d. Cortical bone area. e. Periosteal perimeter. f. Polar moment of inertia. g. Tissue mineral density. Values are shown as mean±SEM, n=10–21 males; 13–19 females. Differences between genotypes were determined by Student's unpaired t-test with Welch's correction. *P<0.05; **P<0.01; ***P<0.001 (*vs.* gender-appropriate wt controls). For dynamic histomorphometric analysis, male femurs were sectioned at the mid-diaphysis; rates were determined at both the endocortex and periosteum. h. Fluorescent images of femur after double-label administration. Representative photomicrographs are shown with higher power insets demonstrating labeling on the endocortex. Bands were photographed at comparable anatomic positions for each bone. i. Bone formation rate (BFR). j. Mineral apposition rate (MAR). k. Percent eroded perimeter. l. Percent mineralizing perimeter (M.Pm/B.Pm×100). Values are shown as mean±SEM; n=8–20 males; 10–15 females. **P<0.01; ***P<0.001 (*vs.* wt controls). Scale bar=200 μm in figure; scale bar=100 μm in insets as indicated.

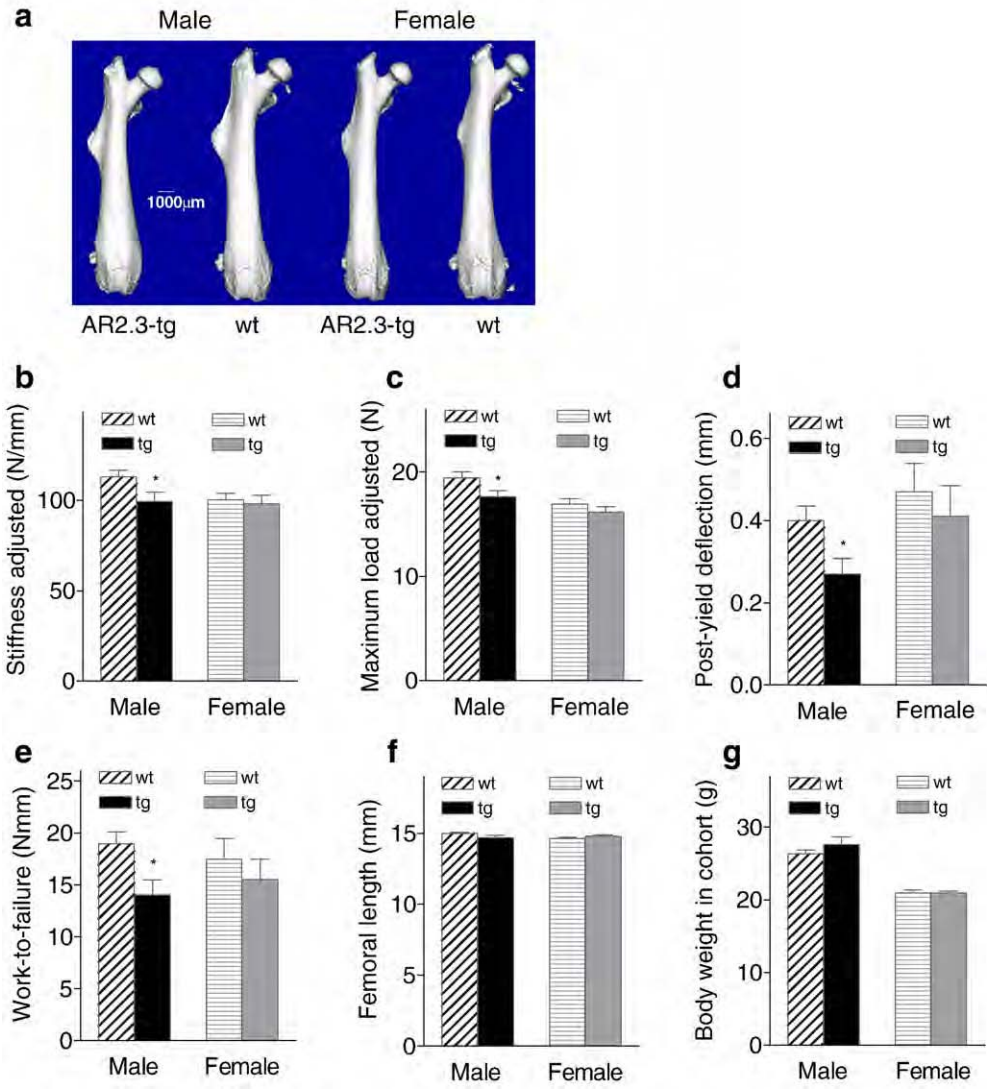

Fig. 4. Whole bone strength and failure properties determined from biomechanical analyses. Femurs from 2-month-old male and female wild-type (wt) and AR2.3-transgenic (tg) mice were loaded to failure in 4-point bending analysis. Stiffness, maximum load, and post-yield deflection were calculated from the load-deflection curves. Stiffness and maximum load are adjusted for body weight differences. a. Whole bone morphology from μCT imaging. b. Stiffness adjusted. c. Maximum load adjusted. d. Post-yield deflection. e. Work-to-failure. f. Femoral length. g. Body weight in cohort. Whole bone biomechanical properties are shown as mean ± SEM, $n = 10–21$ males; 13–19 females. Differences between genotypes were determined by Student's unpaired *t*-test with Welch's correction. $^*P<0.05$ (*vs.* gender-appropriate wt controls).

bone volume was associated with an ~25% increase in trabecular number (Tb.N; $P<0.01$; Fig. 5c), with no effect on trabecular thickness (Tb.Th; Fig. 5d), and thus an ~30% decrease in spacing (Tb.Sp; $P<0.01$; Fig. 5e).

Enhanced androgen signaling in mature osteoblasts leads to reduced expression of molecular markers of bone formation and osteoclast activation in cortical bone

Lastly, we analyzed gene expression in long bone from wild-type and AR2.3-transgenic mice of both genders. Differences in gene expression in RNA isolated from tibial mid-diaphysis for sets of genes that play a significant role in either bone formation or bone resorption (Fig. 6). Osteoblastic marker genes evaluated were cyclin D1, osterix, type I collagen (col), osteocalcin (OC) and sclerostin (SOST). Levels of osteoblastic marker genes are listed in an order reflecting their temporal expression patterns during osteoblast differentiation, *e.g.*, osteocalcin is expressed late in osteoblast differentiation. Significant reductions were noted in transgenic males in levels of osterix, collagen, osteocalcin and SOST (Fig. 6a, left panel). The reduction in osteocalcin gene expression mirrors the reduction in serum concentrations (Fig. 2d). Interestingly, expression of osteoblastic markers in mid-diaphyseal tissue from male transgenic mice was inversely correlated with the differentiation stage, with increased inhibition for genes representative of more mature differentiated osteoblasts, suggesting alteration of the organic matrix consistent with reduced osteoblast vigor shown in Fig. 3j. For example, SOST is produced by mature osteocytes [46], and SOST mRNA levels were significantly inhibited in male transgenic bones. This pattern mirrors the expected increase in col2.3 promoter activity during osteoblast differentiation [12]. Osteoclastic marker genes analyzed were OPG, receptor activator of NF-κB ligand (RANKL), tartrate-resistant acid phosphatase (TRAP), cathepsin K (CatK) and calcitonin receptor (CTR). Similar to the osteoblastic marker genes, inhibition of gene expression was also observed in osteoclastic marker genes CTR, CatK, and TRAP again only in male AR2.3-transgenic mice (Fig. 6a, right panel). The reduction osteoclastic gene expression is consistent with reduced serum levels of bone resorption markers (TRACP5b and CTx; Figs. 2e, f). This qRT-PCR analysis represents changes observed in cortical bone, and may not reflect similar changes in trabecular bone. Consistent with the lack

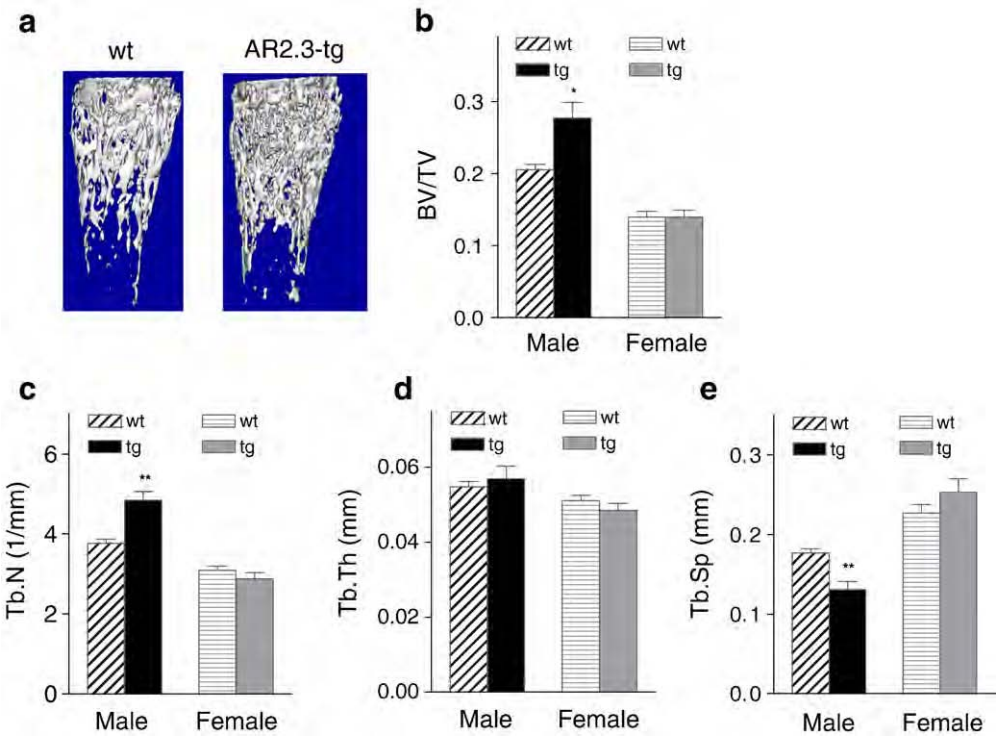

Fig. 5. Trabecular morphology and micro-architecture in AR2.3-transgenic mice. Computer-aided analysis of μCT images was used to derive measures of trabecular bone micro-architecture in the metaphysis of 2-month-old male and female wild-type (wt) or AR2.3-transgenic (tg) mice. Measurements included trabecular bone volume as a percent of tissue volume (BV/TV); trabecular number, spacing, and thickness (Tb.N, Tb.Sp, Tb.Th). a. Reconstructed images were evaluated for trabecular morphology in the distal metaphysis. b. BV/TV. c. Tb.N. d. Tb.Th. e. Tb.Sp. Values are expressed as mean ± SEM, $n = 10–21$ males; 13–19 females. Differences between genotypes were determined by Student's unpaired t-test with Welch's correction. $^{*}P<0.05$; $^{**}P<0.01$ (*vs.* gender-appropriate wt controls).

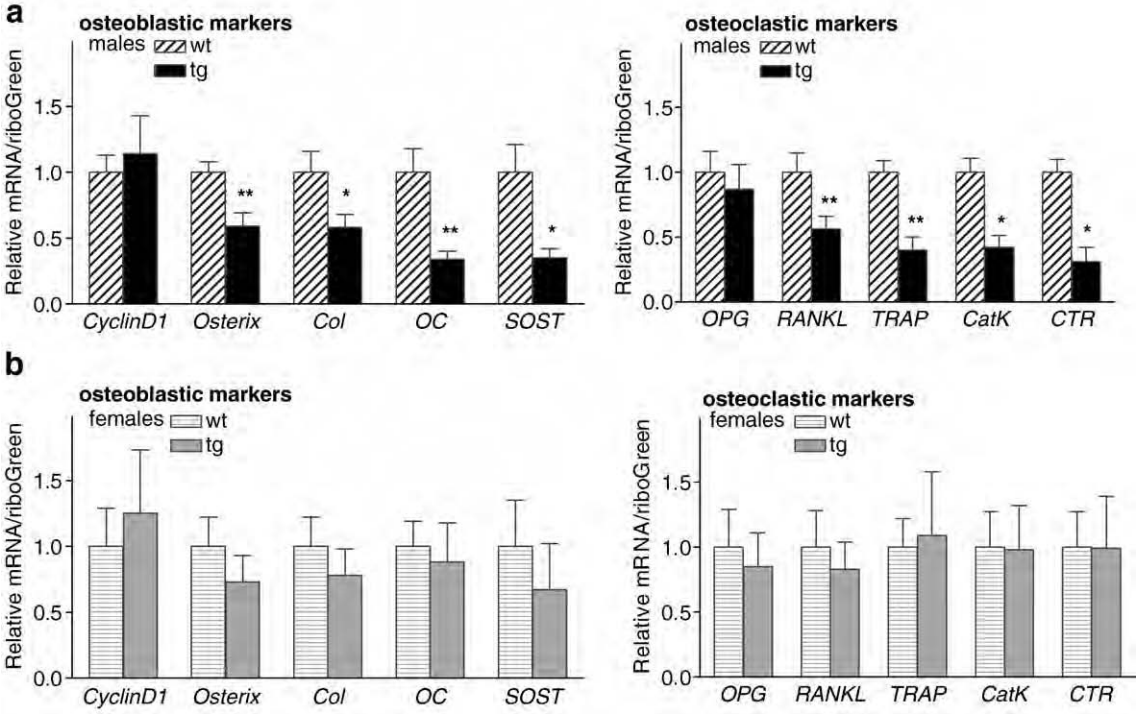

Fig. 6. Cortical bone gene expression in AR2.3-transgenic mice. Analysis of steady-state mRNA expression for genes involved in bone formation or bone resorption was determined by real-time qRT-PCR analysis using tibial RNA isolated from male and female wild-type (wt) or AR2.3-transgenic mice (tg). Osteoblastic marker genes involved in bone formation and matrix production examined included cyclin D1, osterix, type I α_1 collagen (Col), osteocalcin (OC) and sclerostin (SOST). Osteoclastic marker genes involved bone resorption and osteoclastic activity were osteoprotegerin (OPG), RANK ligand (RANKL), tartrate-resistant acid phosphatase (TRAP), calcitonin receptor (CTR) and cathepsin K (CatK). a. Examination of osteoblastic and osteoclastic marker gene expression in male mice. b. Analysis in females. $n = 3–8$ males; 4–7 females. Values are expressed as mean ± SEM. $^{*}P<0.05$, $^{**}P<0.01$.

of a bone phenotype in females, there was little difference in expression in female transgenics compared to control mice for any of the osteoblastic or osteoclastic marker genes analyzed (Fig. 6b).

Discussion

The specific role of androgen signaling though transactivation of the AR in maintenance of skeletal homoeostasis remains controversial. To determine the specific physiologic relevance of androgen action in the mature osteoblast/osteocyte population in bone, mice with targeted AR overexpression in mature osteoblasts were developed. Characterization of the consequences of bone-targeted overexpression revealed a skeletal phenotype in male transgenic mice *vs*. littermate controls, with little difference between the females. Collectively, the phenotype observed in male transgenic mice is likely dependent on the higher serum levels of testosterone (~10-fold) in males *vs*. females. In this study we have found that AR overexpression in the mature osteoblast population *in vivo* results in a low turnover state, with increased trabecular bone volume but a significant reduction in cortical bone area due to inhibition of bone formation at the endocortical surface and a lack of marrow infilling. Combined, our results indicate that AR overexpression in mature osteoblasts/osteocytes inhibits endocortical bone formation, and results in changes that are detrimental to matrix quality, biomechanical competence and whole bone strength.

Among the most striking biomechanical characteristics of the bone from male AR2.3-transgenic mice was its markedly impaired fracture resistance. Brittleness is a measure of the amount of deformation a structure undergoes prior to failure, and long bones were significantly more brittle and consequently showed large decreases in work-to-failure. The observed inhibition in bone quality appears to be principally determined by changes in the organic matrix of bone, through the ~50% reduction in MAR at the endocortical surface (which reflects osteoblast vigor or work). Such a robust reduction would lead to detrimental changes in the composition of the material properties of the organic matrix, and thus a worsening in post-yield deflection and work-to-failure. Brittleness, and its opposite, ductility, are functional attributes that in bone derive principally from matrix composition and collagen organization rather than bone geometry and mass, which are the major determinants of bone stiffness and strength. The increased brittleness (*i.e*., decreased post-yield deflection or ductility) of cortical bone is not likely due to over-mineralization, since TMD was reduced in transgenic males, not increased. Thus, reduced ductility observed in these AR-transgenic mouse bones points to a defect in bone matrix quality. This in turn suggests a defect in osteoblast production of a functionally appropriate bone matrix in the presence of enhanced androgen signaling in mature osteoblasts/osteocytes. Indeed, our molecular analysis of expression differences from the AR-transgenic mice show dramatically reduced collagen and osteocalcin production, consistent with the impaired matrix quality in these mice.

It is instructive to compare the skeletal phenotypes that develop in the two distinct lines that we have generated, the AR2.3-transgenic mice described in these studies and the previously characterized AR3.6-transgenic model [67]. In broad terms, the skeletal phenotype characterized in AR2.3-transgenic mice mirrors that described previously for AR3.6-transgenic males, indicating the reproducibility of the phenotypic consequences of bone-targeted androgen signaling. In common between the two models, we have shown increased trabecular bone volume, reduced formation at endocortical surfaces, reduced bone turnover and compromised biomechanical strength in male transgenic mice. With the exception of enhanced periosteal activity in AR3.6-transgenic males, neither model exhibits anabolic bone formation responses in the cortical bone compartment and instead both show inhibition of bone formation at the endocortical surface. Both also demonstrate significantly compromised biomechanical properties. By comparing and contrasting the two AR-transgenic models, we propose that the commonalities in the bone phenotype between AR2.3-transgenic and AR3.6-transgenic mice arise from AR overexpression in mature osteoblasts and osteocytes, since both promoters are active in these cells. Thus, bone phenotypic changes likely to be mediated at least in part by enhanced androgen signaling in mature osteoblasts/osteocytes include reduced bone turnover, increased trabecular bone volume, reduced endocortical bone formation with decreased osteoblast vigor at endocortical surfaces, and compromised biomechanical strength with increased bone fragility.

The most striking contrast between the two AR-transgenic models is observed at periosteal surfaces in AR3.6-transgenic males, which show increased cortical bone formation in the periosteum and dramatic intramembranous calvarial thickening. This finding was expected, given col3.6 transgene targeting to the periosteum and, conversely, the lack of expression at the same compartment with col2.3 transgene expression [25,26]. The specificity of the periosteal anabolic effect on bone formation in AR3.6-transgenic males is consistent with previous reports documenting the importance of androgen signaling in periosteal expansion [60]. During development, girls and boys build mechanically functional structures (*i.e*., the size, shape and quality of the bone appears to be well-matched for the size of the individual), but by different means [51]. During/after puberty, an increase in estrogen in girls leads to reduced periosteal expansion and then a reversal on the endosteum, from expansion to infilling. In boys, testosterone levels increase, which in contrast to girls is associated with further growth of the periosteum but also continued expansion of the endocortical cavity. Consequently, the outer diameter of girls' bones tends to be smaller than that of boys' bones and greater cross-sectional area is observed in males [13], yet cortical thickness is similar between males and females (but see[41,62]) because of adaptive infilling in females. Thus, we propose that androgen inhibition of medullary bone formation at the endocortical surface in males may subserve an important physiological adaptive function, being the key for appropriate spatial distribution and maintenance of the total amount/weight of bone in the cortical envelope. A reasonable hypothesis is that androgens strongly promote the addition of cortical width through periosteal growth, but balance that growth with inhibition in

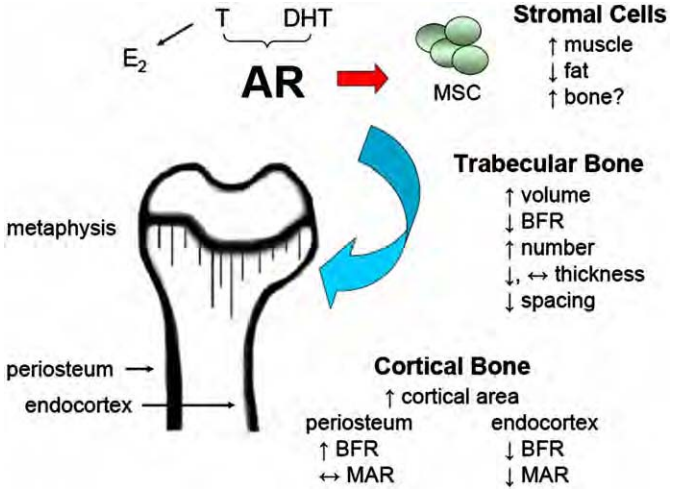

Fig. 7. Model for androgen action in the skeleton mediated by AR transactivation. Androgen activation of AR influences a variety of target organs and skeletal sites, including marrow stromal cells, and trabecular, cortical and intramembranous bone compartments. Arrows indicate the changes associated with androgen action. In trabecular bone, androgen action preserves or increases trabecular number, has little effect on trabecular thickness, and thus reduces trabecular spacing. In cortical bone, AR activation results in reduced bone formation at the endocortical surface but stimulation of bone formation at the periosteal surface. Summary based on results presented here and references cited in the text. In the transgenic model, AR activation in mature bone cells *in vivo* results in a low turnover phenotype, with inhibition of bone formation and inhibition of gene expression in both osteoblasts and osteoclasts. In the absence of compensatory changes at the periosteal surface, these changes are detrimental to overall matrix quality, biomechanics and whole bone strength.

the marrow cavity so that the skeleton does not become too heavy (see [9]). Based on our characterization of AR-transgenic mouse models and other published reports, we propose a model for the consequences of androgen signaling where the effects of AR activation are distinct in different skeletal compartments (Fig. 7). In trabecular bone, androgens reduce bone formation [58] and suppress resorption to increase trabecular volume through an increase in trabecular number. In cortical bone, androgens inhibit osteogenesis at endocortical surfaces but increase bone formation at periosteal sites [67], to maintain cortical thickness yet displace bone further away from the neutral axis in males. Androgens also positively influence bone growth at intramembranous sites [16,67]. In addition to actions directly in bone, androgen administration also increases muscle mass, partially mediated by effects on mesenchymal stem cell lineage commitment [52]. This increase in muscle mass may indirectly influence bone density through biomechanical linkage. Additional studies will be needed to more fully test these hypotheses.

Male AR-transgenic mice also demonstrate a phenotype consistent with reduced osteoclast resorptive activity. In cortical bone, both TRAP and RANKL gene expression is inhibited, and serum levels of both TRAPC5b and CTx are also significantly reduced in transgenic males. In addition, the observed increase in trabecular bone volume with a decrease in trabecular separation is a hallmark of antiresorptive activity. However, future studies employing dynamic histomorphometric analysis will be needed to verify these results. Potential modulation of osteoclast action by DHT is incompletely characterized, although there are reports of AR expression in the osteoclast [57]. Androgen may be a less significant determinant of bone resorption *in vivo* than estrogen [14], but this remains controversial [34]. The bone phenotype that develops in a global AR null male mouse model, a high-turnover osteopenia with reduced trabecular bone volume and a stimulatory effect on osteoclast activity [28,29,69], also supports the importance of androgen signaling through the AR to influence resorption, and is generally opposite to the phenotype we observe with skeletally targeted AR overexpression. Interestingly, the global AR null model also develops late onset obesity [15]. Finally, recent publications document that androgen can directly reduce bone resorption of isolated osteoclasts [45], inhibits osteoclast formation stimulated by PTH [10], and may play a direct role regulating aspects of osteoclast activity in conditional AR null mice [40]. Our results suggest that at least some component of inhibition of osteoclastic resorptive activity as a consequence of androgen administration is mediated indirectly through effects on mature osteoblasts and osteocytes.

Some of the negative consequences of AR overexpression in mature osteoblasts we have observed *in vivo* may reflect previously documented *in vitro* analyses. For example, there are reports, some in clonal osteoblastic cell lines, of effects of gonadal androgen treatment on differentiation, matrix production and mineral accumulation mediated by AR signaling [3,27,53]. These findings are variable however, with other reports of no effect or even inhibition of osteoblast markers [6,18,21], consistent with our gene expression analysis in AR-transgenic mice. In addition, the effect of androgens on osteoblast proliferation is controversial. We have previously demonstrated that either stimulation or inhibition of osteoblast viability by androgen can be observed, and these effects are dependent on the length of treatment. Transient administration of nonaromatizable DHT can enhance transcription factor activation and osteoblast proliferation, while chronic treatment inhibits both mitogenic signaling and MAP kinase activity [66]. Chronic DHT treatment *in vitro* can also enhance osteoblast apoptosis [65]. Combined, these *in vitro* reports are consistent with the detrimental changes in matrix quality and osteoblast vigor we observe in the AR-transgenic model *in vivo*.

In summary, complex skeletal analysis using morphological characterization by μCT, dynamic and static histomorphometric analysis, DXA, biomechanical testing and gene expression studies all indicate that AR overexpression in mature osteoblasts inhibits osteogenesis at endocortical surfaces and produces a low turnover state. Importantly, these changes are detrimental to overall matrix quality, biomechanical competence, bone fragility and whole bone strength. It is possible that the observed inhibition of endocortical osteogenesis and lack of anabolic response, as a consequence of enhanced androgen signaling in mature bone cells, underscores an important physiological function for androgen in the skeleton: to maintain an appropriate spatial distribution of bone in the cortical envelope. Androgens are able to maintain trabecular bone mass and are effective in the treatment of bone loss associated with hypogonadism. Nevertheless, the strong inhibition of bone formation at the endocortical surface and increased bone fragility observed here highlight compartment-specific responses that might underlie the limited therapeutic benefits observed with androgen therapy. Because of the detrimental consequences of bone-targeted androgen signaling on bone fragility and whole bone strength, these results raise concerns regarding anabolic steroid abuse or high-dose androgen therapy during growth and in healthy eugonadal adults.

Acknowledgments

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Abstract No.	Topic
P1-17	Poster abstracts (Authors marked with an asterisk (*) are Recipients of the Alice L. Jee Travel Award)

P-13

Androgen Administration Has Therapeutic Advantages in The Hypogonadal, But Should be Approached With Caution in Healthy Adults

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Androgens are known to have pervasive effects on target tissues including muscle and fat, yet the effects on bone remain poorly characterized. To gain an insight into the cell types important for mediating androgen action, we constructed and compared two distinct transgenic lines of mice employing different α1 (I)-collagen promoter fragments to control skeletally-targeted androgen receptor (AR) overexpression. Histomorphometric and biomechanical analyses revealed compromised bone strength with AR overexpression in bone during development. The role of AR signaling in the adult was characterized *in vivo* using an experimental paradigm of hormone ablation followed by steroid replacement. Control and AR-tg mice were sham operated or gonadectomized at 3 months of age and the effect of nonaromatizable dihydrotestosterone (DHT) was determined after an 8-week delay, allowing for gonadectomy-induced changes to develop.

Following 6 weeks of treatment, the effects of androgen on bone and whole body composition was assessed by DXA. In control mice, systemic DHT administration significantly increased BMD and BMC in both sexes, reversing the loss sustained after a prolonged hypogonadal state. In contrast, in AR-tg mice DHT replacement did not improve either measure compared to placebo. DHT treatment was also beneficial to body composition, improving or fully restoring alterations in lean/fat mass after gonadectomy in control but not AR-tg mice. Further, DHT treatment in intact mice had a negative impact on body composition, reducing lean mass and increasing fat. These findings suggest androgen administration has therapeutic advantages in the hypogonadal, but should be approached with caution in healthy adults.

The authors have no conflict of interest.

Appendix 3:

Zhang X-W, **Wiren KM**: Overexpression of androgen receptor in mature osteoblasts and osteocytes inhibits osteoblast differentiation. (Abstract #SU192) J Bone Miner Res, 2008

Overexpression of androgen receptor in mature osteoblasts and osteocytes inhibits osteoblast differentiation

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Abstract:

Mature osteoblasts/osteocytes are important mediators of androgen action in the skeleton. The direct mechanisms by which androgens influence bone cells are not fully understood. In these initial studies, we have focused on AR2.3-transgenic cultures with overexpression in mature osteoblasts/osteocytes. To assess the direct effects of androgen signaling during osteoblast differentiation, we are studying the effects of DHT on cell differentiation in primary calvarial cultures (mOB) derived from AR2.3-transgenic fetal mice. Osteoblast function is assessed by determining cell growth, alkaline phosphatase activity, and mineral deposition. Osteoblastic cells were isolated after collagenase digestion from neonatal calvaria from both wild-type (wt) and AR2.3-transgenic (AR2.3-tg) mice. Beginning at day 7, cultures were switched to differentiation medium containing 50µg/ml ascorbic acid. From day 14 on, 5 mM ß-glycerophosphate was added to the differentiation media. The mineralization was assessed by alizarin red assay. AR2.3 stable cells were established by co-transfecting with col2.3AR and pRSVneo plasmid DNA in MC3T3-E1 cells under G418 selection. Changes in gene expression (type I collagen and osteocalcin) were determined with qRT-PCR analysis. We first determined the level of AR abundance in both wt and AR2.3-tg cultures by Western analysis, with polyclonal rabbit AR antibodies (ARN-20). As expected, AR levels increase during differentiation in both cultures. The highest level of AR overexpression noted after day 21 in AR2.3-tg cultures. We then assessed growth characteristics of wt and AR2.3-tg cultures without hormone treatment. Osteoblastic cells from both transgenic and wild-type mice display similar growth kinetics. We also determined mineralization capacity, and observed no significant difference between AR2.3-tg versus wt cultures without hormone addition. To assess the consequences of androgen signaling, cultures were treated continuously with vehicle or 10^{-8}M DHT (a nonaromatizable androgen) in charcoal-stripped serum. We characterized DHT action on osteoblast differentiation by determination of alkaline phosphatase (an osteoblast marker activity) and gene expression. Cultures treated with DHT for 14 days showed significant inhibition in alkaline phosphatase activity, with the most robust inhibition observed in AR2.3-tg cultures. Consistent with these results, analysis of gene expression after DHT treatment in stably transfected MC3T3 cultures employing the same promoter showed significant inhibition of both collagen and osteocalcin gene expression. These results are consistent with a reduction in osteoblast differentiation and may reflect the reduced osteoblast vigor observed in AR2.3-tg mice.

Appendix 4:

Semirale A, Wiren KM: DHT Administration Is Effective for the Prevention of Hypogonadal Bone Loss. (Abstract #SU193) J Bone Miner Res, 2008

DHT Administration Is Effective for the Prevention of Hypogonadal Bone Loss

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Abstract:

Generally characterized as anabolic hormones, androgens have pervasive effects on many tissues including bone. Since low bone density and osteoporosis are often coupled with a hypogonadal state in both men and women, sex steroids are implicated in the maintenance of skeletal health. However, the specific effects of androgens on skeletal homeostasis remain poorly characterized and understudied. To gain better insight into the cell types important for mediating the effects of androgens on bone, we constructed and compared two distinct transgenic lines of mice employing different α1(I)-collagen promoter fragments to control skeletally-targeted AR overexpression. The col3.6 AR-transgenic (AR3.6-tg) mice demonstrate AR overexpression throughout the osteoblast lineage including the periosteum, while col2.3 (AR2.3-tg) mice have more restricted overexpression in mature osteoblasts and osteocytes. To assess the effect of the non-aromatizable androgen DHT on bone mineral, we compared a treatment versus prevention experimental paradigm in a rodent model of hypogonadal bone loss. AR -transgenic mice and wild-type B6D2F2 littermates were gonadectomized or sham operated, receiving subcutaneous DHT pellet implants either at the time of surgery or after a two month delay. Following six weeks of DHT administration, bone mineral was assessed by whole body DXA. In the treatment model, after two months in a hypogonadal state, wild type mice of both genders lose significant BMD and BMC. In this setting, six weeks of DHT treatment was effective at partially restoring lost bone mineral. In contrast, while both AR2.3-tg and AR3.6-tg male and female mice similarly lost BMD and BMC, they were resistant to the effects of DHT treatment over the same time frame. In the prevention model, during a high bone turnover state, both male and female wild type mice were protected from bone mineral loss when DHT was present compared to placebo controls. AR3.6-tg and AR2.3-tg male mice were also protected from bone loss following gonadectomy and AR3.6-tg males even gained BMD and BMC with six weeks of DHT administration. In contrast, female AR2.3-tg and AR3.6-tg mice did not benefit and continued to lose bone mineral in the presence of DHT. In summary androgen therapy may be beneficial for the prevention of hypogonadal bone loss, but is less effective at restoring lost bone.